The role of dopaminergic projections from the ventral tegmental area to the nucleus accumbens core and shell in responding to a discrete alcohol cue in different contexts

Milan David Valyear

A Thesis
In the Department
Of
Psychology

Presented in Partial Fulfillment of the Requirements
For the Degree of
Doctor of Philosophy (Psychology) at
Concordia University
Montreal, Quebec, Canada

August 2020

CONCORDIA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

This is to certify that the thesis prepared

By: Milan D. Valyear

Entitled: The role of dopaminergic projections from the ventral tegmental area to the nucleus accumbens core and shell in responding to a discrete alcohol cue in different contexts

and submitted in partial fulfillment of the requirements for the degree of

Doctor Of Philosophy (Psychology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

_____ Chair
Dr. Richard Courtemanche

_____ External Examiner
Dr. Rutsuko Ito

_____ External to Program
Dr. Alisa Piekny

_____ Examiner
Dr. Uri Shalev

_____ Examiner
Dr. Peter Shizgal

_____ Thesis Supervisor
Dr. Nadia Chaudhri

Approved by _____
Dr. Andrew Chapman, Graduate Program Director

August 21, 2020

_____
Dr. André Roy, Dean
Faculty of Arts and Science

ABSTRACT

The role of dopaminergic projections from the ventral tegmental area to the nucleus accumbens core and shell in responding to a discrete alcohol cue in different contexts

Milan D. Valyear, Ph.D.
Concordia University, 2020

Environmental stimuli that predict alcohol are powerful triggers for relapse in humans. These stimuli become established as alcohol cues through Pavlovian conditioning and can be grouped into discrete cues (e.g. smell, sight, taste of alcohol) and contexts (e.g. bar). In the current thesis, a Pavlovian conditioning with context alternation procedure was used to measure the capacity for an alcohol-associated context (alcohol context) to influence responding to a discrete alcohol conditioned stimulus (CS). Responding to a discrete alcohol CS was elevated in an alcohol context compared to an equally familiar neutral context. By incorporating other behavioural procedures, the influence of context over responding to a discrete alcohol CS was shown to be persistent and temporally sensitive.

Pharmacology and chemogenetic approaches revealed that dopamine neurotransmission and the activity of ventral tegmental area (VTA) dopamine neurons was necessary for responding to a discrete alcohol CS in a neutral context. Further, chemogenetic inhibition of the dopaminergic projection from the VTA to the nucleus accumbens (NAc) core reduced CS responding in the alcohol and neutral context. Differently, chemogenetic inhibition of the dopaminergic projection from the VTA to the NAc shell selectively reduced CS responding in the alcohol context, and not in the neutral context. Thus, the dopaminergic VTA-to-NAc core projection was necessary for responding to a discrete alcohol CS whereas the dopaminergic VTA-to-NAc shell projection was necessary for the elevation of this behaviour in an alcohol context.

Targeting of tyrosine hydroxylase (TH) positive VTA dopamine neurons and the use of chemogenetics to manipulate neural activity in a circuit-specific manner were validated using immunocytochemistry and electrophysiology, respectively. Chemogenetic designer receptors were selectively (~95%) expressed in TH positive VTA neurons. Chemogenetic inhibition or excitation of VTA dopamine terminals in the NAc modulated the amplitude of electrically-evoked excitatory postsynaptic currents in NAc core medium spiny neurons (MSNs). Thus, a circuit-

specific chemogenetic approach could be used to target VTA dopamine neurons and bidirectionally affect the activity of postsynaptic MSNs.

Based on the finding that the VTA-to-NAc shell projection was necessary for the elevation of CS responding in the alcohol context, the sufficiency of activating VTA dopamine neurons and their projection to the NAc shell to drive CS responding was tested. Chemogenetic activation of VTA dopamine neurons failed to impact responding to a discrete alcohol CS in a neutral context, despite affecting feeding behaviour in the same rats. Further, chemogenetic activation of the dopaminergic VTA-to-NAc shell projection failed to affect CS responding in the alcohol and neutral context. Thus, chemogenetic activation of VTA dopamine neurons or their projections to the NAc shell was not sufficient to elevate responding to a discrete alcohol CS.

The lack of an effect of activating dopaminergic substrates on responding to a discrete alcohol CS suggested that non-dopaminergic circuits are recruited to elevate responding to a discrete alcohol CS in an alcohol context. This hypothesis was examined by microinfusing a glutamate-like agonist in the NAc shell during tests for CS responding in the neutral and alcohol context. Responding to a discrete alcohol CS was selectively reduced by microinfusion of a glutamate-like agonist in the alcohol context and was unaffected in the neutral context. This selective effect on CS responding in the alcohol context implicates glutamatergic activity in the NAc shell in the influence of context over responding to a discrete alcohol CS.

In summary, responding to a discrete alcohol CS is elevated in an alcohol context and the influence of context over this behaviour is persistent and temporally sensitive. Responding to a discrete alcohol CS requires the activity of VTA dopamine neurons and their projections to the NAc core. The elevation of responding to a discrete alcohol CS in an alcohol context requires the dopaminergic projection from the VTA to the NAc shell. Attempts to elevate responding to a discrete alcohol CS by activating dopaminergic or glutamatergic substrates either failed to affect or reduced CS responding, respectively. Thus, the circuits sufficient to elevate responding to a discrete alcohol CS may involve the coordinated activation of dopaminergic and glutamatergic inputs to the NAc shell. Ultimately, a detailed description of the circuits that mediate the influence of discrete cues and contexts over responding for alcohol will inform the development of efficacious behavioural and pharmacological therapies for alcohol use disorder.

ACKNOWLEDGEMENTS

Firstly, I would to thank my supervisor, Dr. Nadia Chaudhri who provided me with the opportunity to pursue a PhD in the beautiful city of Montréal. She introduced me to new and exciting science and fostered my curiosity with frequent and fun discussions. Nadia's encouragement, thoughtful mentorship, and careful approach to science continue to shape my endeavours in science and life. I am forever grateful for the time I spent in the Chaudhri Lab. Thank you, Nadia.

I am indebted to the members of the thesis committee. Dr. Rutsuko Ito, Dr. Alisa Piekny, Dr. Uri Shalev, and Dr. Peter Shizgal. Thank you for serving as members of my committee. I also thank Dr. Richard Courtemanche for serving as chair and organizing the defense. I am grateful for the efforts of the committee to produce a careful examination of my thesis and provide critical feedback, especially under exceptional circumstances (COVID-19).

The Center for Studies in Behavioral Neurobiology (CSBN) was a wonderful place to be. I had the privilege of interacting with Isabelle Bouvier, Steve Cabilio, Dave Munroe, and Heshmat Rajabi on a frequent basis, and relied on their careful assistance to guide me through various challenges. Thank you all.

I would be remiss if I did not mention the exceptional collegiality at the CSBN. I was lucky to work with many talented colleagues who were kind enough to share their knowledge. Almost all of the techniques I used in my thesis were taught to me by Dr. Firas Sedki, Dr. Tracy D'Cunha, Dr. Gonzalo Quintana-Zunino, Dr. Czarina Evangelista, Dr. Nuria Zavalia, Pavel Solis, Dr. Waqqas Shamms, and Ariel Batallán Burrowes. During my time at the CSBN, Aileen Murray was the manager of the animal facility and in addition to ensuring that our animals were in good health, she took the time to teach me a number of basic neuroscience techniques.

I owe a special thank you to Dr. Peter Shizgal, Dr. Ivan Trujillo-Pisanty, and Dr. Marie-Pierre Cossette for countless science discussions and for beginning the TH::Cre breeding colony at Concordia. Ivan and Marie taught me many important techniques, talked me through complex ideas, and became great friends throughout the process.

Dr. Andrew Chapman and Dr. Iulia Glovaci were wonderful collaborators and conducted a critical experiment in this thesis. It was a pleasure to work with such thoughtful scientists.

Dr. Uri Shalev frequently asked me how things were going, and brief hallway-discussions often informed my experimental designs. Thank you being approachable and helpful, Uri.

The Chaudhri lab at the CSBN was packed with skilled and supportive lab-mates. Franz Villaruel is an exceptional lab-mate and friend; we had so much fun. Franca Lacroix helped me get acquainted with the lab when I first started and is one of the most effective teachers I have ever known. Mandy Lecocq consistently produced exceptionally good behavioural data and was always willing to offer tips. Alexa Brown joined the lab towards the end of my graduate studies, but we shared many laughs in the lab and quickly became good friends. I also enjoyed working with Audrey Zaari, Soraya Lahlou, Nadine Padillo, Ghislaine Deyab, Rami Khouri, and Nina Caporicci-Dinucci who were remarkable undergraduate students with an inspiring interest in neuroscience.

Being a graduate student at Concordia, and particularly in Nadia's lab, made it very easy to interact with the science community in Montréal. I was incredibly fortunate to talk with Dr. Jane Stewart about my research on a number of occasions, she helped me with a draft of one of my papers and to write my thesis – Thank you Jane! I have fond memories of the Drugs, Brain, and Behaviour Journal Club organized by Dr. Paul Clarke, where I presented and received feedback on my research. Through the public scholars program at Concordia, and with the help of Fiona Downey and Edie Austin, I was able to publish an Op-Ed in the Montréal Gazette which was a wonderful experience.

Lastly, I would like to thank my family and friends. My parents Luisa and Timothy Valyear encouraged my curiosity and taught me the value of perseverance. My sisters Arisa and Kiara Valyear offered sage advice and often uplifted me with witty jokes. My grandparents Fortunato and Rose Fusco taught me the importance of ingenuity and served as a constant source of inspiration. My two childhood best friends, Adam Giacomazzo and Philip Rossi always made me feel like I was making them proud and this support was so important – thank you to you both. My partner Tara Delorme coached me through a number of challenges that seemed impossible at the time and I couldn't have done this without her constant support.

Finally, this thesis was supported by grants from CIHR (MOP-137030, Nadia Chaudhri), FRQS (Chercheur Boursier Junior 2, Nadia Chaudhri), CFI (John R. Evans Leaders Fund, Nadia Chaudhri) and NSERC (2017-04802, Nadia Chaudhri C; 2014-0507, C. Andrew Chapman).

Milan D Valyear was supported by a Helene Linder Doctoral Fellowship, a Faculty of Arts and Science Graduate Fellowship, and a Concordia Public Scholar Award.

CONTRIBUTION OF AUTHORS

Chapter 1:

Experiment 1a, b and c are published under a Creative Commons v4.0 International License which permits redistribution of the material in any medium or format. Experiments 1a, b and c appear in Valyear, M. D. *et al.* Dissociable mesolimbic dopamine circuits control responding triggered by alcohol-predictive discrete cues and contexts. *Nat. Commun.* **11**, 3764 (2020).

Experiment 2 is published under Copyright by Elsevier B.V. which grants authors permission to include article content in theses and dissertations. Experiment 2 appears in Valyear, M. D. & Chaudhri, N. Context controls the timing of responses to an alcohol-predictive conditioned stimulus. *Behav. Processes.* **173**, 104061 (2020).

-Milan Valyear and Nadia Chaudhri conceptualized and designed experiments 1a, b, and c.
-Milan conducted experiments 1 a, b, and c, analyzed the data, and created the figures.
-Nadia Chaudhri contributed to the analysis strategy and figure creation.
-Milan Valyear designed, conducted, analyzed the data, and created the figures for experiment 2.
-Nadia Chaudhri contributed to the figure design and analysis strategy for experiment 2.

Chapter 2:

All experiments in chapter 2 are published under a Creative Commons v4.0 International License which permits redistribution of the material in any medium or format. All experiments in chapter 2 appear in Valyear, M. D. *et al.* Dissociable mesolimbic dopamine circuits control responding triggered by alcohol-predictive discrete cues and contexts. *Nat. Commun.* **11**, 3764 (2020).

-Milan Valyear bred and genotyped all the TH::Cre rats for all experiments in chapter 2 with assistance from Ivan Trujillo-Pisanty, Audrey Zaari and Soraya Lahlou.
-Milan Valyear and Nadia Chaudhri conceptualized and designed experiments 1, 2, 3, 4, and 5.
-Milan Valyear and Ivan Trujillo-Pisanty conceptualized and designed experiment 6.

-Iulia Glovaci and C. Andrew Chapman designed and conceptualized experiment 7.
-Milan Valyear and Nadia Chaudhri contributed to the conceptualization of experiment 7.
-Milan Valyear collected, analyzed the data, and generated the figures for experiments 1, 2, 3, 4, 5 and 6.
-Nadia Chaudhri contributed to the analysis strategy and figure design for experiments 1, 2, 3, 4, 5 and 6.
-Audrey Zaari assited with data collection for experiments 2 and 3.
-Soraya Lahlou assited with data collection for experiment 3.
-Milan Valyear conducted the surgeries and contributed to the figure creation for experiment 7.
-Iulia Glovaci conducted the electrophysiological recordings, analyzed the data, and created the figures for experiment 7.
-C. Andrew Chapman contributed to the analysis strategy and figure creation for experiment 7.

Chapter 3:

Experiment 4 in chapter 3 was published under a Creative Commons v4.0 International License which permits redistribution of the material in any medium or format. Experiments 4 appears in Valyear, M. D. *et al.* Dissociable mesolimbic dopamine circuits control responding triggered by alcohol-predictive discrete cues and contexts. *Nat. Commun.* **11**, 3764 (2020).

-Milan Valyear bred and genotyped all the TH::Cre rats for all experiments in chapter 2 with assistance from Soraya Lahlou, and Ghislaine Deyab.
-Milan Valyear and Nadia Chaudhri designed and conceptualized experiments 1, 2, 3 and 5.
-Iulia Glovaci and C. Andrew Chapman designed and conceptualized experiment 4.
-Milan Valyear and Nadia Chaudhri contributed to the conceptualization of experiment 4.
-Milan Valyear collected, analyzed the data, and generated the figures for experiments 1, 2, 3, and 5.
-Nadia Chaudhri contributed to the analysis strategy and figure design for experiments 1, 2, 3, and 5.
-Soraya Lahlou assisted with data collection for experiments 1 and 2.
-Ghislaine Deyab assisted with data collection for experiments 3 and 5.
-Alexa Brown and Nina Caporicci-Dinucci assisted with data collection for experiment 3.

-Milan Valyear conducted the surgeries and contributed to the figure creation for experiment 4.

-Iulia Glovaci conducted the electrophysiological recordings, analyzed the data, and created the figures for experiment 4.

-C. Andrew Chapman contributed to the analysis strategy and figure creation for experiment 4.

General Discussion:

Some of the arguments that appear in the discussion about context serving as an occasion setter were published under Copyright by Elsevier B.V. which grants authors permission to include article content in theses and dissertations. These arguments appear in Valyear, M. D., Villaruel, F. R. & Chaudhri, N. Alcohol-seeking and relapse: A focus on incentive salience and contextual conditioning. *Behav. Processes* **141**, 26–32 (2017).

-Milan Valyear, Franz Villaruel, and Nadia Chaudhri all contributed to the writing and formulation of arguments in this manuscript.

Table of Contents

Lists of Figures

Chapter 1: The extent to which context influences responding to a discrete alcohol CS

Chapter 2: The necessity of separable VTA dopamine circuits for responding to a discrete alcohol CS in different contexts

Chapter 3: The role of dopaminergic and glutamatergic activity in responding to a discrete alcohol CS in different contexts

“The unfamiliarity of the surroundings had a big effect upon the animal; it shivered slightly, and became as though spellbound.”

Ivan P. Pavlov, 1927, p. 268

General Introduction

Overview

The transition from controlled to problematic alcohol use is often preceded by exposure to environmental stimuli that were present during previous alcohol use events[1,2]. Environmental stimuli become established as cues that predict alcohol through repeated pairings with the pharmacological effects of alcohol. The capacity for alcohol cues to provoke alcohol use is evidenced by self-report studies in which people list the types of cues that trigger a craving for alcohol[3]. Further support are studies in which the desire to consume alcohol is shown to increase in the presence of a discrete alcohol cue, like a wine bottle[4], or after experiencing an alcohol context, like a bar[5]. In real-world drinking scenarios, discrete alcohol cues and alcohol contexts co-occur, yet little is known about how the concomitant presentation of these cues influence behaviour, and much less is known about the neural circuits that govern this influence. The aim of the current thesis is to contribute to the understanding of how discrete alcohol cues and contexts interact to encourage responding for alcohol and identify the neural circuits that support this behaviour.

In chapter 1 the principal behavioural procedure for the thesis, whereby the influence of an alcohol context over responding to a discrete alcohol cue is measured, was described. Briefly, rats received Pavlovian conditioning sessions wherein a discrete auditory conditioned stimulus (CS) predicted the delivery of alcohol for oral consumption in a distinct alcohol context. Pavlovian conditioning sessions were alternated with sessions of exposure to a neutral context wherein alcohol was never available. After an equal number of sessions in both contexts, conditioned responding to the discrete alcohol CS was tested by presenting the CS without alcohol in the alcohol and neutral context on separate days. Also in chapter 1, the persistence of CS responding in extinction and the capacity for an alcohol prime to reinstate responding in the alcohol and neutral contexts, was examined. Furthermore, the capacity for context to control the timing of responses elicited by a discrete alcohol CS was explored. It is argued from the results of these studies that responding to a discrete alcohol CS is persistently elevated in an alcohol context relative to a neutral context and that context has the capacity to control nuanced properties of the responses elicited by a discrete alcohol CS.

In chapter 2, the involvement of the dopamine system and its projections to the nucleus accumbens (NAc) core and shell in responding to a discrete alcohol CS in different contexts

was investigated. Specifically, the necessity of the dopamine system and dopaminergic neurons in the ventral tegmental area (VTA) for responding to a discrete alcohol CS was examined using pharmacology and chemogenetics. Next, the role of the dopaminergic projections from the VTA to the NAc core and shell in responding to a discrete alcohol CS in both the neutral and alcohol context was explored using a circuit-specific chemogenetic approach. The circuit-specific inhibitory chemogenetic approach was validated using electrophysiology and immunocytochemistry. Collectively, the results from chapter 2 demonstrated that responding to a discrete alcohol CS required the activity of VTA dopamine neurons and their projections to the NAc core, whereas the elevation of responding to a discrete alcohol CS in an alcohol context required the dopaminergic projection to the NAc shell. Chapter 2 thus outlined a point of separability in the dopaminergic circuits that support responding to a discrete alcohol cue and the elevation of this behaviour in an alcohol context.

In chapter 3, the sufficiency of activity in the dopamine system and NAc shell to elevate responding to a discrete alcohol CS was examined. Using chemogenetics, VTA dopamine neurons and their projections to the NAc shell were excited to test whether this activation was sufficient to elevate responding to a discrete alcohol CS, however CS responding was unaffected in these experiments. In subsequent experiments using the same rats, the capacity for chemogenetic excitation of VTA dopamine neurons to affect behaviour was confirmed using a feeding assay. The excitatory circuit-specific chemogenetic approach was also validated using electrophysiology. Next, to test whether general excitation of the NAc shell impacted responding to a discrete alcohol CS, a glutamate-like agonist was microinfused into the NAc shell before tests in both the alcohol and neutral contexts. Unexpectedly, increasing NAc shell activity selectively reduced responding to the discrete alcohol CS in the alcohol context, but not in the neutral context. Chapter 3, suggested that while VTA dopamine neurons and their projections to the NAc shell may be necessary for responding to a discrete alcohol CS and the elevation of this behaviour by context (chapter 2), respectively, chemogenetic activation of these substrates was not sufficient to elevate responding to a discrete alcohol CS. Further, the effects of glutamatergic activity in the NAc shell on responding to a discrete alcohol CS suggested that the activity sufficient to elevate responding to a discrete alcohol CS may involve multiple neurotransmitter systems.

Together, the behavioural data from chapter 1 generated new information about the capacity for contexts to influence responding to a discrete alcohol CS while the pharmacology

and chemogenetic experiments in chapters 2 and 3 begin to identify the neural underpinnings of responding to a discrete alcohol CS in and outside of an alcohol context. An alcohol context can control the magnitude, persistence, and timing of responses elicited by a discrete alcohol CS. Further, the dopaminergic projection from the VTA to the NAc core is necessary for responding to a discrete alcohol CS independent of context. Alternatively, the dopaminergic projection from the VTA to the NAc shell is necessary, but not sufficient, for the elevation of responding to a discrete alcohol CS in an alcohol context. Lastly, increasing glutamatergic activity within the NAc shell disrupted responding to a discrete alcohol CS in an alcohol context, further demonstrating the involvement of the NAc shell in responding that is influence by context. Collectively, this thesis contributes to the understanding of how discrete cues and contexts interact to influence responding for alcohol and identify dissociable dopamine circuits that underlie this influence.

The role of context in Pavlovian conditioning

In the early 20th century, Ivan P. Pavlov and his collaborators demonstrated that initially innocuous stimuli could come to provoke complex physiological and behavioural responses[6]. This phenomena, now termed Pavlovian conditioning, occurs when an initially neutral stimulus (NS) comes to elicit a conditioned response (CR) through its predictive relationship with an unconditioned stimulus (US), and is thus established as a conditioned stimulus (CS)[6]. In this influential work the CS took many forms (e.g. metronome, light, buzzer, whistle, etc.) and occurred for durations of seconds to minutes depending on the experiment. Often, CSs predicted the delivery of food reinforcers to be consumed by the subjects and produced a conditioned salivation response. The capacity of a CS to elicit a CR through Pavlovian conditioning is a remarkably well-replicated and robust phenomenon, with entire scientific societies[7] dedicated to studying this type of learning. Further, many contemporary learning phenomena, such as extinction, overshadowing, summation, and reinstatement can be traced back to work by Pavlov and his collaborators[8,9]. Despite the existing depth of knowledge about Pavlovian conditioning, many questions remain. One question that is of particular relevance to this thesis is: how does the context in which Pavlovian conditioning occurs affect the development and expression of the CR?

The work conducted by Pavlov and his collaborators used exclusively brief CSs to establish the principles of Pavlovian learning and this practice set a precedent that was perpetuated by future researchers. The importance of the learning context, however, was not

lost upon Pavlov. In the chapter on Hypnotic Stages, in *Conditioned Relfexes* Pavlov detailed the story of a dog that was trained to salivate in response to a discrete CS; this particular dog had never rejected the food reinforcer and was brought outside the lab, in front of an audience for a demonstration of the conditioned salivation and consummatory responses. “On administration of the conditioned stimulus the normal secretory effect was obtained, but the dog did not take the food, and in a relatively short time fell into profound sleep in its stand, right in front of the audience, with complete relaxation of the skeletal muscles” (Pavlov, 1927, p. 268)[6*]. While the salivation response was maintained in this new context, the consummatory response was interrupted and an unexpected narcoleptic response emerged for the first time. This brief passage demonstrates that the elicitation of an established conditioned response depends critically upon the context in which the CS is presented.

Contexts differ in modality and duration from traditional CSs that are commonly used in Pavlovian conditioning procedures. Traditional CSs, often termed discrete CSs or cues, are brief in duration and reliably predict the US by gaining prominence during the US. Contexts, however, occur stably in the background, are often multimodal, and persist for long periods of time in the absence of the US. Therefore, the opportunity exists for CS-US pairings to become embedded within the training context, and for context to become integral for the expression of learning.

Context can influence how animals respond to discrete CSs. Transswitching is a conditioning procedure whereby a discrete CS, termed a *phasic* stimulus in this literature, is presented against a background of *tonic* stimuli (different chambers, experimenters, time of day, etc.) and differentially reinforced depending on the properties of the tonic stimuli[10,11]. Asratyan (1965) described a series of experiments using transswitching in dogs in which the same discrete CS predicted a US that differed in size (food), temporal relation to the CS, or valence (food vs shock) depending upon the background of tonic stimuli. In these experiments the tonic stimuli came to control the magnitude, timing, and valence of responses elicited by the discrete CS. These results led Asratyan to theorize that tonic stimuli “…which are at first neutral, gradually become *constantly acting conditioned stimuli which can evoke conditioned reflexes of a tonic type*, and create a definite functional background or readiness for phasic activity” (Asratyan, 1965, p. 137)[10]. This theory of tonic conditioned reflexes suggested that tonic stimuli, which are akin to contexts, dictated *what* US will occur, whereas the discrete CS dictated *when* the US will occur[12]. The recognition that tonic stimuli, or contexts, could be established as

[*] The quotation by Pavlov that opens this thesis directly precedes this sentence.

conditioned stimuli marked an important theoretical advance of focusing not only on discrete CSs in Pavlovian learning, but also on the contexts in which discrete CSs occur.

The work on transswitching demonstrated that context exhibits exquisite control over responses that are elicited by a discrete CS, however the mechanisms through which context controls CS responding is debated[13]. Both discrete cues and contexts can intricately control behaviour and a deeper understanding of the theoretical and neurobiological underpinnings of this control is warranted to build upon the early transswitching studies.

In addition to the reasons suggested by learning theory, there is an applied reason for studying the influence of context on responding to discrete CSs. Substance use disorders are characterized as chronically relapsing disorders with relapse rates across alcohol, tobacco, cocaine, and opiates ranging from 65% to 98% depending on the drug class and treatment received[14,15]. Often, contexts and discrete cues associated with drug use are reported as triggers for relapse[1,3] and alcohol consumption is elevated in alcohol contexts (e.g. bar)[16–18]. The focus of the current thesis is to understand the role of context in controlling responses to a discrete alcohol CS and to describe the neural circuits that govern responding that is influenced by discrete CSs and contexts.

Understanding the capacity of discrete cues and contexts to influence alcohol-seeking and consumption, is particularly relevant for developing efficacious behavioural therapies for alcohol use disorder. A goal of cue-exposure therapy is to extinguish the reactivity (e.g. craving, desire to drink) that people experience when presented with alcohol cues[19,20]. It stands to reason, that the efficacy of cue-exposure therapy is determined by the similarity between the cues used in therapy, and the cues encountered in real-world drinking scenarios. For example, one of the most potent cues for relapse are the orosensory and visual properties of alcohol itself[2,21]. Conducting cue-exposure therapy involving limited exposure to alcohol in a context wherein alcohol was previously consumed would most adequately resemble a real-world drinking scenario and possibly improve therapeutic efficacy. However, providing alcohol to be consumed by patients with alcohol use disorder is ethically challenging, despite the implication that it might aid in the efficacy of cue-exposure therapy. One study allowed in-patients to participate in cue-exposure therapy sessions that involved consuming and interacting with alcohol beverages while trying to resist further consumption[22]. Remarkably, the capacity for patients to resist drinking increased across sessions and their desire do drink decreased; both

of these measures differed significantly from a control group that had only imagined drinking scenarios. While this study does not examine the impact of a real-world alcohol context on the efficacy of cue-exposure therapy, it confirms that learning to resist the urge to consume alcohol in highly realistic drinking scenarios greatly improves the efficacy of cue-exposure therapy.

Animal models of relapse

Operant models of relapse

Environmental stimuli that reliably predict substance use become established as Pavlovian cues that instigate drug-seeking and -taking[23]. When questioned about the types of stimuli that trigger a craving for alcohol people report a variety of stimuli that might be considered discrete cues (TV commercials, ice cold drink on poster) or contexts (cocktail parties, bars)[3]. Further, presentations of alcohol cues can influence people to transition to earning alcohol for later consumption in a task where participants have free choice between alcohol or money[2]. As such, animal models of relapse have attempted to capture the power of these cues to trigger drug-seeking behaviour.

The original investigations into the capacity for cues to potentiate or cause drug-seeking to re-emerge focused on discrete cues. Davis and Smith (1976) showed that rats would lever-press to earn intravenous infusions of morphine that occurred concomitantly with a buzzer (discrete cue)[24]. The operant response was then extinguished by replacing the morphine solution with saline and discontinuing buzzer presentations for a few sessions. After lever-pressing had reached low levels in extinction, reintroducing the buzzer contingent upon lever-presses caused responding to become significantly elevated despite morphine being unavailable. This basic procedure and finding details the cue-induced reinstatement procedure that is often used in modern preclinical research[25,26]. Harriet de Wit and Jane Stewart demonstrated a similar re-emergence of responding after extinction of lever-pressing for cocaine upon the reintroduction of a tone that was paired with cocaine delivery earlier in the same session[27]. Building on this set of studies[28], de Wit and Stewart demonstrated that non-contingent infusions of drugs with similar stimulus properties could also reinstate lever-pressing for a drug after extinction. In other words, stimulants were much more efficacious at triggering the reinstatement of lever-pressing for stimulants than were opiates, and the inverse. The finding that drugs of similar classes were most efficacious at reinstating responding after extinction suggested that the reactivation of similar neurotransmitter systems contributed to relapse, and further that neutral stimuli might come to activate these neurotransmitter systems

through parings with the pharmacological effects of drugs. Altogether, these findings led to an important theoretical proposition by Stewart and colleagues; that the perpetuation of substance use in humans is maintained by the conditioned stimuli that are paired with unconditioned drug effects because they come to generate motivationally relevant states and elicit drug-taking actions[23†].

Although early studies on the cue-induced reinstatement model of relapse focused on opiate and stimulant drugs, this phenomena has also been demonstrated with alcohol in dependent[29] and non-dependent rats[29–31], and mice[32]. The preclinical evidence that discrete alcohol cues influence alcohol-seeking behaviour echoes findings from the human literature showing that discrete alcohol cues can elicit conditioned responses. For example, when people handle and interact with an alcohol-associated beverage (e.g. cocktail) they experience changes in skin-conductance, pulse, and pleasure rating compared to a non-alcohol-associated beverage (e.g. soft drink)[33]. Further, deliberate pairings of neutral flavours with alcohol in humans can condition changes in skin-conduction, pulse, and ratings of arousal[34]. Importantly, the finding that physiological responses and arousal can be deliberately conditioned, indicates that similar responses are likely conditioned in real-world drinking scenarios in which the properties (e.g. sight, smell, taste) of certain alcohol beverages reliably predict the pharmacological effects of alcohol.

The cue-induced reinstatement model of relapse captures many interesting features of substance use in humans; however, there are at least two features of this model that warrant further consideration. In the cue-induced reinstatement procedure the discrete cue is always presented in a response-contingent manner. The re-emergence of lever-pressing after extinction, which is thought to model relapse, is produced by lever-presses that trigger the presentation of discrete cues[25]. In a real-world setting, a person attempting to abstain from substance use may be prompted to resume substance use when confronted with discrete drug cues. However, in the cue-induced reinstatement procedure, the subject must earn the presentation of those initial drug cues by lever-pressing, which seems unlike the real-world corollary. An additional difficulty of response-contingent cue presentation emerges when considering the psychological processes that govern cue-induced reinstatement. It has been

† The idea that conditioned stimuli were critical for maintaining substance use stood in stark contrast to drive-reduction[232] views of substance use which suggested that relapse was perpetuated by craving or withdrawal symptoms that manifested over time. This review by Stewart et al., (1984) marked a turning point in the dominant thinking about substance use disorder from a drive-reduction framework to a conditioning framework.

documented in the literature previously that rats will acquire lever-pressing responses for the presentation of naturally reinforcing stimuli such as brief flashes of light[35–37]. Therefore, when responding re-emerges in the reinstatement test it is unclear whether that behaviour is motivated by the naturally reinforcing properties of discrete stimuli or the acquired reinforcing properties of the stimuli that have been achieved through association with the primary drug reinforcer. These two caveats complicate the interpretation of the psychological and neurobiological processes that operate during cue-induced reinstatement.

While discrete cues can powerfully influence alcohol and drug-seeking in animals and humans, context also influences relapse-like behaviours[38]. People report an increased craving for alcohol and consume more alcohol when they experience a bar context relative to an neutral environment (e.g. office or library)[5,39,40]. As well, a preference for an otherwise neutral context can be conditioned by allowing people to consume alcohol or methamphetamine in that context[41,42]. Furthermore, patients suffering with alcohol use disorder often report contexts (e.g. bars or social settings) as events that precipitate relapse and generate an urge to drink[1,3].

Much of the work incorporating context manipulations into animal models of relapse was derived from studies on fear conditioning which showed that learning about the relationship between a discrete CS and shock was context specific. For example, conditioned freezing that was established through pairing a discrete CS with an aversive shock in a specific training context was extinguished by presenting the CS without shock in a different context, and renewed by placing the subject back into the original training context[43]. Also, conditioned freezing to a discrete CS that had undergone acquisition and extinction in a similar context was reinstated following un-signaled delivery of a shock in the same context, but not in a different context[44]. These studies, which were Pavlovian in nature, led researchers to theorize that learning about a discrete CSs depended upon the stimuli present in the background, and ultimately suggest that context modulates responding to discrete CSs[45].

Contexts associated with drug use produce a renewal of responding in animals that is thought to model relapse[46,47]. In the original report of the context renewal relapse model, rats were trained to press a lever to earn the concomitant presentation of a discrete cue and a drug reinforcer, which was a *speedball* mixture of cocaine and heroin[46]. After lever-pressing behaviour stabilized, the rats were placed into a different context defined by olfactory, tactile, visual, and temporal stimuli, to receive extinction sessions wherein lever-presses earned

presentations of the discrete cues but not the drug reinforcer. During this extinction phase lever-pressing decreased to low levels over the course of a few sessions, at which point a renewal test was conducted by returning the rats to the original training context. Upon return to the original training context, lever-pressing was renewed to comparable levels as before extinction despite drug being unavailable. Importantly, rats that remained in the extinction context, or were placed into a novel context, for the renewal test did not show a renewal of responding. In sum, the context renewal procedure demonstrates the capacity for a drug-associated context to cause a re-emergence of extinguished drug-seeking behaviour, which may resemble a relapse episode in humans.

The context renewal procedure has been well-replicated across a variety of drug reinforcers[48] and spawned new models which incorporate punishment or alternative rewards during the extinction phase[48–50]. Importantly, context renewal has been demonstrated with alcohol as the reinforcer in rats[51–53]. Also, a modified version of the context renewal procedure has been reported in people, in which salivation and urge to consume in response to a discrete alcohol cue was conditioned in a particular room, extinguished in a different room, and renewed upon return to the original room[54]. The demonstrations of context renewal with alcohol in humans and rats affirms that context has the capacity to control responding that is motivated in part by discrete cues. Further, the context renewal procedure demonstrates some tractability in terms of studying the influence of context over responding for discrete alcohol cues.

The renewal model captures an important property of relapse in humans in that contexts are frequently reported as relapse triggers[1], however there are at least two features of the renewal model than warrant further consideration. The first feature is that during the renewal test the discrete cues and the context are presented concurrently. This produces some difficulty in interpreting the psychological mechanism through which context causes a re-emergence of responding. For example, context may influence the processing of discrete drug cues whereby discrete cues resume the capacity to support drug-seeking behaviour. Alternately, context may influence responding directly by generating a relevant motivational state. Finally, context may operate through some combination of multiple processes. When multiple psychological processes adequately explain a behavior, as is the case for responding in the renewal test, it is difficult to associate a neurobiological mechanism to that behavior. For example, if inhibiting a particular neural substrate reduced responding in the renewal test, it could be concluded with near-equivalent likelihood that this substrate was necessary for responding to discrete cues,

contexts, or their combination. The second feature worth considering is that in the renewal model, context is presented in a non-contingent manner; this stands in contrast to the cue-induced reinstatement procedure in which cue presentations are response-contingent. That context is presented non-contingently is not a caveat of the renewal procedure itself, but creates a difficulty in uncovering the psychological and neurobiological underpinnings of relapse triggered by discrete cues and contexts[32]. For example, if a certain neurobiological manipulation produced different behavioural consequences in the cue-induced reinstatement and renewal procedures then this dissociation could be attributed to differences in contingent versus non-contingent cue presentation, or differences between discrete cues and contexts. Altogether, a careful analysis of the psychological processes that may operate during the renewal test reveals that it is difficult to parse the contributions of discrete cues and context to behaviour using the context renewal model.

Pavlovian models of relapse

The theory from which investigations of the influence of cues over drug-seeking behaviour and relapse stems is that environmental stimuli become established as drug-cues through Pavlovian conditioning. Paradoxically however, Pavlovian models of relapse emerged more recently in history than did operant models of relapse. A major advantage of Pavlovian models of relapse is that discrete cues can be presented in combination with, or in isolation from, contexts associated with a drug reinforcer, which permits study of the combined and independent influence of discrete drug cues and contexts on responding. Separating the influence of context and discrete cues over responding in an operant procedure is difficult because the operant, often a lever, may be considered a discrete cue, particularly when it is inserted into the chamber on separate trials. A second advantage of Pavlovian models of relapse is that discrete cues and contexts are presented in a response non-contingent manner, whereas in operant models discrete cues are presented response-contingently and contexts are presented non-contingently. These two advantages are critical to associate the activity of a particular neural substrate with responding for a drug reinforcer that is influenced by discrete cues, contexts, or their combination.

Chaudhri et al, (2008) used a Pavlovian conditioning procedure to demonstrate that an alcohol-associated context could renew responding to a discrete alcohol CS[55]. In this study, rats underwent Pavlovian discrimination training in a distinct alcohol context wherein two discrete stimuli (white noise or clicker) were presented. One stimulus designated the CS+ predicted the

delivery of alcohol into a fluid port for oral consumption, whereas the other stimulus served as a CS- that was presented without alcohol. After responding to the CS+ was acquired, rats underwent extinction in a distinct context. After extinction, rats received a renewal test session in the alcohol context wherein the CS+ and CS- were presented without alcohol. Responding to the CS+ was renewed upon return to the alcohol context. Importantly, these results demonstrated that an alcohol-associated context could renew Pavlovian responding to a discrete alcohol cue and described a basic framework for conducting a Pavlovian renewal procedure with alcohol.

A subsequent investigation used a similar Pavlovian discrimination training procedure and context manipulations to delineate the contributions of nucleus accumbens subregions to responding elicited by a discrete alcohol cue in an alcohol or neutral context[56]. In a first set of experiments rats implanted with cannulae targeting either the nucleus accumbens (NAc) core or shell received Pavlovian discrimination training in a particular alcohol context. Extinction sessions occurred in a second distinct context and then renewal tests were conducted by returning the rats to the original alcohol context. Before renewal tests in the alcohol context, rats received microinfusions of saline or muscimol/baclofen in a within-subjects design to pharmacologically inactivate the NAc core or shell. Inactivation of either subregion reduced context renewal indicating that these subregions were necessary for the renewal of responding to a discrete alcohol CS. In a second set of experiments, rats similarly underwent Pavlovian discrimination training, and then responding to the discrete CS was tested in a neutral context in which rats had never received alcohol. In this neutral context test, pharmacological inactivation of the NAc core reduced CS responding, whereas inactivation of the NAc shell had no effect on responding. Thus, the NAc core and shell were similarly necessary for Pavlovian context renewal but were disparately involved in responding to a discrete cue in a neutral context.

Comparing the results of NAc core and shell inactivation in the context renewal tests and the neutral context tests, generated some insight into how the NAc core and shell are involved in responding to a discrete alcohol cue, and responding that is influenced by context. The necessity of the NAc core for responding in the renewal model and in the neutral context test indicated that the NAc core was necessary for responding to a discrete alcohol CS, which was common to both procedures. Differently, that inactivation of the NAc shell reduced responding to the discrete CS in the renewal test wherein responding re-emerged in the alcohol context, but not in the neutral context test, suggested that the shell was necessary for responding that was

influenced by context. Together these results suggested that the NAc core was necessary for responding to a discrete alcohol CS whereas the shell was important for responding that was influenced by context, however, differences between the two procedures complicate this interpretation. For example, only the context renewal procedure involved extinction sessions prior to test, whereas the neutral context tests were preceded by exposure sessions to the neutral context. Thus, NAc shell inactivation may have attenuated the renewal of responding by interfering with the expression of extinction learning, or by interfering with the processing of relevant contextual information.

Pavlovian conditioning with context alternation and alcohol-seeking circuits

Previous efforts to delineate the contributions of contexts and discrete CSs to responding for alcohol, and further to describe the neurobiology underlying these contributions, contain caveats that limit their interpretation. To circumvent these limitations, Sciascia et al, (2015) devised a completely within-subjects protocol wherein contexts and discrete cues were presented non-contingently, the overall amount of exposure to an alcohol-associated context and a neutral context was equated, and neither context had a training history involving extinction[57]. In this protocol Pavlovian conditioning sessions, wherein a discrete auditory CS was paired with alcohol delivery into a fluid port, were conducted every other day in an alcohol context that was defined by olfactory, visual, and tactile stimuli. On the intervening days, rats were exposed to a distinct neutral context wherein alcohol was never available. After an equal number of training sessions in both contexts, tests were conducted by presenting the discrete CS in both contexts without alcohol. Rats made significantly more port entries during the CS in the alcohol context than in the neutral context at test[57]. This finding demonstrated that an alcohol-associated context had the capacity to elevate responding elicited by a non-extinguished discrete alcohol CS. Importantly, this report outlined a procedure in which both discrete cues and contexts are presented non-contingently, the alcohol and neutral contexts are equally familiar, and avoided a training history including extinction in either context.

Preclinical studies have implicated the NAc in Pavlovian and operant responding for alcohol, however the identity of the inputs that are relevant for these effects remains elusive. The NAc, consisting primarily of medium spiny neurons (MSNs) that release γ-aminobutyric acid, is densely innervated by dopaminergic inputs from the midbrain[58] and glutamatergic inputs from cortical areas[59]. Glutamatergic inputs to the shell arrive from a variety of cortical areas including the basolateral amygdala (BLA)[59–64] and ventral subiculum (vSub) of the

hippocampus[59,62–65]. Differently, dopaminergic inputs to the NAc arrive exclusively from the ventral tegmental area (VTA), within which cells near the midline project predominantly to the NAc shell, and more lateral areas project to the NAc core[66–68]. Both dopaminergic and glutamatergic inputs to the NAc appear necessary for responding for alcohol. For example, pharmacological inactivation of the BLA or vSub reduced context renewal of Pavlovian[69] and operant[70] responding for alcohol, respectively. Similarly, dopamine antagonists microinfused to the NAc core and shell reduced context renewal of alcohol-seeking[71] and systemically administered dopamine antagonists reduced the context renewal of Pavlovian responding for alcohol[72]. Thus, critical hubs within the glutamatergic and dopaminergic neurotransmitter systems emerge as important for Pavlovian and operant responding for alcohol.

The procedure used by Sciascia et al., (2015) and the finding that an alcohol-associated context elevated responses elicited by a discrete alcohol CS, has been replicated[73] and used to probe the brain regions that support this effect[57]. Reducing glutamatergic neurotransmission in the BLA attenuated CS responding (i.e. port entries during the CS) in both the alcohol and neutral contexts[57] whereas pharmacological inactivation of the BLA selectively reduced CS responding in the alcohol but not neutral context[73]. Interestingly, overall activity in the NAc shell was necessary for CS responding in the alcohol context but not the neutral context[73]. It is perhaps not surprising that both glutamatergic and dopaminergic systems have both been implicated in Pavlovian responding for alcohol. Glutamatergic and dopaminergic inputs converge onto the same MSNs in the NAc[74], and glutamate and dopamine levels in the NAc are covary[75–78]. Thus, studies using the Pavlovian conditioning with context alternation procedure underscore a role for the NAc and suggest that the core and shell subregions play dissociable roles in controlling this behaviour.

The role of mesolimbic dopamine in responding to discrete cues and contexts

Dopamine cells in the ventral tegmental area (VTA) densely innervate the NAc[58,68] and this projection has been has been implicated in alcohol use disorder. For example, the consumption of alcohol and presentation of alcohol cues modulates dopamine levels in the accumbens of rats[79,80] and people[81,82], and the dopamine system is dysregulated in people suffering from alcohol use disorder[83,84]. Further, systemically administered dopamine antagonists reduced responding in operant and Pavlovian models of alcohol relapse[53,71,72,85]. The involvement of the dopamine system in preclinical models of relapse suggests that dopaminergic projections to the NAc core and shell may in part underlie their separable roles in

responding that is motivated by discrete cues and contexts. Additionally, the dichotomous involvement of the NAc core and shell in responding to different types of environmental stimuli is supported by observational studies that measured the activity of these subregions during behaviour[86,87].

Perhaps, the most obvious reason to suggest that separable neural circuitries underlie the influence of contexts and discrete cues on behaviour, is that discrete cues and contexts evoke different types of behaviours. Responses elicited by discrete cues are often temporally delimited and further the pursuit or consumption of a reinforcer. Alternatively contexts, particularly when paired with drugs that act on the mesolimbic dopamine system, can induce a place-preference[88], facilitate sexual behaviour [89,90], and increase locomotor activity[91–94] in rats. Importantly, all of these context effects are conditioned and demonstrate a capacity for context to influence diverse forms of behaviour presumably through neural circuits that differ from the circuits that control the typically brief responses that are elicited by discrete CSs.

The hypothesis that dopaminergic projections underlie dissociable functions of the NAc core and shell is supported by studies comparing the effects of microinfusing a dopamine D1-like antagonist into these subregions in operant renewal and reinstatement models of heroin relapse[95]. Microinfusing the D1-like antagonist into the NAc core failed to affect responding in the context renewal procedure wherein the return of responding was motivated by context, whereas the same microinfusion in the NAc shell attenuated the renewal of responding. Interestingly, responding at test in the reinstatement procedure, in the absence of a context manipulation, was unaffected by microinfusing the D1-like antagonist into the shell whereas the same microinfusion in the core attenuated responding. These results suggest that dopaminergic projections to the core and shell underlie the separable involvement of these subregions in responding that is influenced by contexts and discrete cues, although these interpretations should be made cautiously. In the renewal procedure both discrete cues and contexts are presented concurrently, meaning that interfering with neural circuits that support the processing of discrete cues, contexts, or their association may all reduce responding. Additionally, comparing findings from the reinstatement and renewal models is complicated because discrete cues are presented contingently upon responding in both models whereas context is presented noncontingently in the renewal model. Therefore, the presentation of discrete cues and contexts is confounded by contingent, and noncontingent cue presentation, respectively. Lastly, in the reinstatement procedure, responding at test is reinforced by presentations of a tone-light cue

which may be naturally reinforcing[35–37]. Thus, reductions in responding in the reinstatement procedure may be caused by perturbations of circuits that support the naturally reinforcing properties of the stimulus, the secondary reinforcing properties attributed to the stimulus through its associated with heroin, or both.

The suggestion that the NAc core and shell are disparately involved in responding to different types of environmental cues is also supported by observational studies that measure dopamine release in these regions during operant responding for a food reward[86,87]. In a chained-sequence of 'seeking' and 'taking' lever-insertions and presses for a food reward, dopamine release in the nucleus accumbens core tacked closely with earliest reliable predictor of reinforcement whereas release in the shell tracked equally all components of the chain-sequence[‡], but waned throughout the session[86]. Dopamine release in the core might encode the predictive relevance of a cue which would explain why core, but not shell, lesions impair the acquisition of cocaine self-administration when rats must earn presentations of cocaine-cues before infusion lever-presses are reinforced[96]. While dopamine release in the core aligns strongly with optimal predictors of reinforcement and wanes thereafter, release in the shell is pronounced during reinforcer consumption and cues that are poor predictors of reinforcement but motivationally salient due to their proximity to reinforcer consumption[86,87,97]. Further evidence that the shell tracks motivational salience is that throughout a session, while rats became sated and presumably less motivated to earn food presentations, the shell response became blunted and correlated with longer latencies to respond[86]. Thus, dopamine release in the core is tightly linked to the predictive value of a cue whereas dopamine release in the shell might reflect motivational salience.

The theoretical interpretation that NAc core dopamine encodes the predictive value of a cue, whereas NAc shell dopamine encodes motivational salience supports the dissociation that these subregions are disparately involved in responding that is influenced by discrete cues and contexts. In real-world drinking scenarios the orosensory properties of a beverage reliably

[‡] It is worth noting that the stability of the NAc shell dopamine response throughout various aspects of the chain sequence[86] aligns with a prediction made by Asratyan[10] and other transswitching researchers[11] about the neural signatures that could represent a tonic stimulus or context. In one experiment, Asratyan trained a dog to receive a shock in one context (morning) on the right hindleg during a discrete CS, and on the left hindleg in a different context (afternoon). Using crude electroencephalogram technology of the day, Asratyan discovered a stable increase in activity of the anterior parietal cortex ipsilateral to the shocked limb that persisted throughout the entirety of sessions in both contexts. This finding was the first confirmation that brain activity underlying the influence of context on behaviour might represent a relatively stable and persistent change in activity, like NAc shell dopamine in the Saddoris et al., (2015) study.

precede consumption whereas the ambience of a bar is stably present in the background during and outside of consumption events. Preclinical models of relapse capture this feature of real-world drinking because discrete cues are established as the best predictors of reinforcement whereas motivationally relevant contexts persist stably in the background during drug consumption, but also during relatively long periods when the drug is unavailable (e.g. inter-trial intervals, pre- and post-session delays). Therefore, it is reasonable to predict that responding to a discrete cue which has high predictive value depends more heavily on dopaminergic activity in the NAc core than shell. Conversely, a motivationally relevant context, that is a poorer predictor of reinforcer delivery may preferentially recruit NAc shell dopamine circuits that encode motivational salience.

It may seem strange to suggest that alcohol-associated contexts influence responding for alcohol through mechanisms other than their direct predictive association with reinforcer identity, but there is support for this idea from human studies in which participants are asked about their knowledge of a conditioned stimulus. In the conditioned place preference assay, a preference for a particular context is generated because of that context's association with unconditioned drug effects. Conditioned place preference for alcohol has been demonstrated in mice[98–100], rats[101,102], and humans[41]. Although consuming alcohol in a particular room generated a preference to spend more time in that room in people, only ~10% of the participants reported an awareness of the association between the preferred context and alcohol, and the place preference was maintained when these participants were excluded from the analysis[41]. This finding demonstrates that context can influence behaviour through affecting the motivational salience of a context, in the absence of a conscious association between context and alcohol.

The dopamine system, and particularly dopaminergic activity in the NAc core and shell have been implicated in behaviour that is controlled by drug-associated discrete cues and contexts. The suggestion that NAc core dopamine is necessary for responding to discrete cues whereas NAc shell dopamine supports responding that is influenced by context, aligns with the theoretical interpretation that dopaminergic activity in the NAc core and shell encode predictive value and motivational salience, respectively. Additionally, VTA dopamine as the source for this core shell dichotomy is supported by the finding that the firing rates of VTA dopamine neurons in response to an aversive discrete cue can be modulated by the motivational value of the context in which the discrete cue is presented[103]. Therefore, the proposal is made in the current thesis that the dopaminergic projection from the VTA to the NAc core is necessary for

responding to a discrete alcohol cue in a context-independent manner, whereas the parallel projection to the NAc shell supports responding to a discrete cue in the presence of an alcohol context.

Chapter 1: The extent to which context influences responding to a discrete alcohol CS

Introduction

Environmental stimuli that reliably predict alcohol become established as Pavlovian alcohol cues that instigate the pursuit and consumption of alcohol[2–4]. Discrete alcohol cues are brief environmental stimuli that gain prominence during alcohol consumption (e.g. sight, smell, taste)[3,57,104]. Alcohol-associated contexts are a stable configuration of environmental stimuli that are present in the background during alcohol consumption. Discrete alcohol cues and contexts co-occur when alcoholic beverages are consumed in an alcohol-associated context, like a bar[3,57,104,105]. Deciphering the extent to which responding to a discrete alcohol cue is influenced by an alcohol context is critical to understand the psychological processes that engender alcohol cues with the capacity to affect behaviour. The experiments in this chapter used two Pavlovian conditioning procedures to characterize the influence of context on responding to a discrete alcohol cue. It was found that an alcohol context persistently elevated responses triggered by a discrete alcohol cue and that context has the capacity to control nuanced properties of the response form.

Using a Pavlovian conditioning procedure, Chaudhri and her collaborators have shown that responses to a discrete alcohol cue are significantly elevated in an alcohol-associated context relative to an equally familiar context that has no association with alcohol[57,73]. In this procedure, rats receive Pavlovian conditioning sessions wherein a discrete auditory conditioned stimulus (CS) is paired with alcohol in a distinct context, referred to as the 'alcohol context,' every other day. On the days intervening these sessions rats are exposed to a different 'neutral context' without alcohol. After an equal number of sessions in both contexts, CS responding (port entries during the CS) is tested by presenting the CS without alcohol in both contexts. Typically, CS responding is elevated in the alcohol context relative to the neutral context. An important feature of this Pavlovian conditioning with context alternation procedure is that the familiarity of both the alcohol and neutral contexts is equated prior to test, which improves upon earlier procedures that blocked context exposures into separate phases of varying durations[104,106].

One caveat of Pavlovian conditioning with context alternation as used previously is that the alcohol and neutral contexts differed in terms of acoustical salience during training. Pavlovian conditioning sessions in the alcohol context included a discrete auditory stimulus, the CS, whereas only controlled background noises were present during sessions in the neutral

context. The volume of static background noise and the frequency of discrete stimulus presentations can sensitize or habituate a startle response to a discrete auditory stimulus[107]. Therefore, experiencing an acoustically impoverished neutral context during training may have attenuated responding to the CS at test in the neutral context, which is the first time a discrete auditory stimulus is presented in this context. Another possibility is that presentations of the auditory CS in the alcohol context during training may have been more arousing and sensitized responding at test. In experiment 1a this possibility was addressed by including a discrete auditory neutral stimulus (NS) in the neutral context during training in one group of rats and comparing their behaviour to a group that received only controlled background noises in the neutral context (**Fig 1c, 2a**).

Previous studies using Pavlovian conditioning with context alternation demonstrated that CS responding was elevated in the alcohol context relative to the neutral context but did not examine whether this effect was transient, or persistent[57,73]. Interestingly, these previous reports discovered that while responding waned across CS trials during single test sessions, CS responding was stably elevated in the alcohol context relative to the neutral context throughout the test session. The stability of this context-induced elevation within a test session suggests that the influence of context over responding to a discrete CS may be long-lasting and persist across multiple test sessions. In experiment 1b the persistence of responding to a discrete alcohol CS in the alcohol and neutral context was examined by conducting multiple repeated test sessions (**Fig 3a**) in both contexts and observing the trajectory with which responding extinguished.

Following the repeated test sessions, experiment 1c was conducted to examine whether the capacity for context to influence CS responding was retained after extinction (**Fig 3c**). After responding had diminished to similar levels in both contexts, prime-induced reinstatement tests were conducted by delivering a drop of alcohol for oral consumption at the beginning of an otherwise unreinforced session. Delivering a drop of alcohol to reinstate responding after extinction has been used as a model of relapse previously in operant[108] and Pavlovian conditioning procedures[109], and an alcohol-prime can increase alcohol consumption in humans[2,21]. This prime-induced reinstatement test also expanded the investigation of the persistence of the contextual control of responding to a discrete alcohol CS to include a model of relapse.

Context can serve as a trigger for relapse and drug-seeking[3,110], and if context encodes when a drug becomes available in relation to a discrete CS then this information could influence the development, expression, and re-emergence, of drug-seeking behaviours. CS onset itself can signal when a US may occur. For example, short intervals (1-5 s) between CS onset and a food unconditioned stimulus (US) produced immediate salivation in dogs, whereas salivation was delayed with longer intervals between the CS and US[6]. Unlike a discrete CS, physical contexts persist stably in the background during learning, and little is known about the capacity of context to signal the temporal relation between a CS and US[111–113]. It appears that only one study has investigated this question, and found that salivation to a CS in dogs occurred sooner when the CS was presented in a context wherein food was delivered earlier during the CS, compared to a different context where food was delivered later during the same CS[10].

Experiment 2 was conducted to investigate whether context could signal the temporal relation between CS onset and an alcohol unconditioned stimulus (US). A Pavlovian conditioning procedure was developed in which rats were trained in two different contexts on alternating days according to a within-subjects design. In one context, called the *early* context, alcohol was delivered from the onset of the 5th second until the termination of the 10th second of a 30 s CS. In a different *late context*, alcohol was delivered from the onset of the 25th second until the termination of the 30th second of the same CS (**Fig 4a**). It was predicted that in trained rats the probability of making a conditioned response in the first four seconds of the CS – an interval that preceded US onset in both contexts – would be higher in the early context than the late context.

In summary, experiment 1a was conducted to replicate previous findings that an alcohol context elevated responding to discrete alcohol CS, while addressing the concern of having an acoustically impoverished neutral context during training. Experiment 1b extended previous work by assessing the persistence of elevated CS responding in the alcohol context across repeated test sessions and in a reinstatement model of relapse. Finally, experiment 2 tested the capacity for context to control the timing of responses to a discrete alcohol CS.

Methods

Subjects

Male, Long-Evans rats (n=24) were ordered from INVIGO Laboratories, Indianapolis, USA and weighed 220-275 g on arrival (Exp. 1) or were bred in-house (n=12) on a mixed

Charles River and INVIGO background (Exp. 2). All rats were individually housed in standard polycarbonate shoebox cages (20 x 24 x 45 cm) and maintained on a 12 h light-dark cycle (lights on at 0700) at 21±2°C at 40-50% humidity. All procedures were conducted in the light phase and rats had unrestricted access to chow (Charles River Rodent Diet #5075), tap water, and a nylabone™ chew-toy. All experimental procedures complied with the Animal Research Ethics Committee at Concordia University and the Canadian Council on Animal Care regulations.

The final number of rats included in experiment 1 was n=22, due to a programming error in one conditioning chamber. On the first day of training this conditioning chamber was designated the alcohol context for one rat in each of the CS/NS and CS/NoNS groups. This means that the final sample size for the CS/NS and CS/ NoNS groups was 11 rats/group. However, because this chamber was an alcohol context on the first training session for two rats, the group sizes for the reinstatement test were 10 for the alcohol context and 12 the neutral context.

Behavioral apparatus

Behavioral training and testing occurred in 12 conditioning chambers (ENV-009A; Med-Associates Inc.), enclosed in fan-ventilated (~77 dB), sound-attenuating, melamine cubicles (53.6 x 68.2 x 62.8 cm). The right wall featured a fluid port (17.5 cm from rear wall, 9 cm from front door) that contained two wells (ENV-200R3AM). Fluid delivery into one well occurred through a 20 ml syringe attached to a pump (PHM-100, 3.33 rpm) located outside the cubicle. Fluid-port entries were measured with an infrared beam (ENV-205M) and recorded to a computer using Med PC-IV software, which also controlled fluid delivery and stimulus presentations. The upper left wall featured a clicker stimulus (ENV-135M, 8 dB above background), a continuous white noise stimulus generator (ENV-225SM, 8 dB above background), and a white house-light (ENV-215M).

Solutions and reagents

Odours (10% concentration, v/v) were prepared by adding lemon oil (SAFC Supply Solutions, St-Louis, MO, USA; #W262528) or benzaldehyde (almond odor; OMEGA Chemical Company Inc., Levis, QC, Canada; #B37-50) to tap water. Alcohol (15% ethanol, v/v, room temperature) was prepared weekly by diluting 95% ethanol in tap water.

General behavioural procedures

Experiment 1. The persistence of elevated CS responding in the alcohol context

Home-cage alcohol exposure

Imposing intermittent schedules of alcohol availability has been shown to produce gradual elevations in alcohol consumption[106,114–119]. All rats received 24 h access to alcohol and tap water every other day for 12 sessions, over 24 days (**Fig 1a**). On the intervening days, only water was available. Alcohol was provided in a 100 ml graduated cylinder fitted with a rubber stopper containing a sipper tube with a metal ball bearing to minimize spillage. Alcohol cylinders and water bottles were placed onto opposite sides of a standard cage lid and weighed before and after every 24 h session. The position of the water bottle and ethanol cylinder was switched at the beginning of every session to control side-preferences. A filled alcohol cylinder and water bottle were placed onto two empty cages and weighed to monitor spillage. The average spillage as a result of handling the cylinder and bottle was subtracted from each rat's daily consumption. Rats were weighed every other day before receiving alcohol cylinders.

Habituation

On the last day of home-cage alcohol exposure, rats were brought to the behavioural testing room in their home-cages and individually handled. On the two subsequent days all rats were habituated to Context 1 and then 24 h later to Context 2 in the conditioning chambers. Context 1 consisted of black walls, a clear Plexiglas floor, and a lemon odour. Context 2 consisted of clear Plexiglas walls, a wire-mesh floor, and an almond odour. Odours (3 sprays) were applied to a petri dish placed in the waste-pan under the chamber floor. Entries into a fluid port in the conditioning chamber were recorded during each context habituation session. All habituation sessions were 20 min long. Behaviour room and conditioning chamber habituation sessions occurred at the same time of day as subsequent training and test sessions.

Pavlovian conditioning with context alternation

Rats were assigned to the CS/NS or the CS/NoNS group based on their final home-cage alcohol consumption (g/kg) to create two balanced groups. Rats were then assigned to receive Pavlovian conditioning sessions in Context 1 or 2 to equate home-cage alcohol consumption within each condition.

Pavlovian conditioning sessions (12 sessions; 73.5 min each) were similar for both the CS/NS and the CS/NoNS groups and occurred in a distinct context called the 'alcohol context'

every other day including weekends. Immediately after the rats were placed into their respective conditioning chambers the MED-PC program was initiated. Each conditioning session began with a two-minute delay during which port entries were recorded. After the delay the house-light was illuminated, and the first inter-trial interval (ITI) began. During each session 15 trials of an auditory CS (10 s; white noise or clicker) occurred on a variable-time (VT) 260 s schedule, with ITIs of 140, 260, or 380 s. With every CS presentation 0.2 ml of alcohol was dispensed into the fluid port over the last 6 s of the CS, resulting in a total 3 ml of alcohol delivered per session. Ports were checked at the end of each session to make sure all the alcohol was consumed.

On the days intervening the Pavlovian conditioning sessions all rats were exposed to a distinct 'neutral context' for 73.5 min sessions. The neutral context was the same conditioning chamber in which Pavlovian conditioning occurred but was configured as a different context type. In other words, rats assigned to receive context 1 as the alcohol context experienced context 2 as the neutral context, and the inverse. The house-light was illuminated after a similar two-minute delay in the neutral context, but the remainder of the session differed between the CS/NS and the CS/NoNS groups. For the CS/NS group an auditory NS (clicker or white noise) that was different from the assigned CS, was presented on a 260 s VT schedule but alcohol was never delivered. For the CS/NoNS group, the house-light was illuminated, and only control background noises were present for the duration of the neutral context sessions. Port entries made in the neutral context during the NS or immediately preceding the NS (PreNS) were used in analyses but for the CS/NoNS group these corresponding intervals were empty and termed No Neutral Stimulus (NoNS) or Pre No Neutral Stimulus (PreNoNS). For both groups, the syringe pumps were activated on a similar schedule as during Pavlovian conditioning sessions, but syringes were not placed on the pump. This alternating context training procedure was used to equate the frequency of exposure to both contexts throughout training while still allowing the rats to discriminate between the alcohol and neutral contexts (**Fig 1c**).

Experiment 1a. CS responding tests in the alcohol context and neutral context

Following training, CS responding was tested in the alcohol context and neutral context (**Fig 2a**). Rats then had two re-training sessions, one per day in each context, prior to a second test that occurred in the opposite context. At test, the CS was presented on the same VT 260 s schedule as training, but alcohol was not delivered. Syringe pumps were activated as during training. Half of the rats in each group received the CS responding test in the alcohol context first, and the remainder in the neutral context.

Experiment 1b. Repeated test sessions in the alcohol context and neutral context

Next, repeated tests were conducted to examine the persistence of elevated CS responding in the alcohol context relative to the neutral context. Starting 24 h after the second test all rats were given repeated daily tests by presenting the CS, without alcohol, in both the alcohol and neutral contexts on alternating days for a total of eight sessions (four per context; **Fig 3a**). Half of the rats received the first repeated test session in the alcohol context and the other half received the first repeated test in the neutral context. In each repeated test session, the CS was presented on a VT 260 s schedule, but alcohol was not delivered. Syringe pumps were activated as during training.

Experiment 1c. Reinstatement in the alcohol context or neutral context

Lastly, the capacity of context to influence the return of responding after extinction was examined in a prime-induced reinstatement procedure. After responding had decreased to similarly low levels in both contexts all rats received a reinstatement test in either the alcohol context (n=10) or neutral context (n=12), according to a between-subject design (**Fig 3c**). Starting 24 h after the last repeated test session the rats that began the repeated test session phase in the neutral context received the reinstatement test in the alcohol context and the inverse. To trigger reinstatement at test a 0.2 ml drop of alcohol was dispensed over 6 s into the fluid port at 30 s into the 2 min pre-session delay to serve as a reminder of the taste and smell of alcohol. A second 0.2 ml drop of alcohol was delivered during the last 6 s of the first CS presentation, but alcohol was withheld for the remainder of the test.

Experiment 2. The capacity for context to control the timing of CS responses

The rats from experiment 2 were first used in a pilot study to examine if different access schedules impacted the voluntary consumption of alcohol. Briefly, three groups of rats received access to alcohol in their home-cage for 24 h sessions that occurred every day (n=4), every other day (n=4), or every fourth day (n=4) over 45 days[106,118,119]. Alcohol intake (g/kg) increased over sessions [Session, $F_{(11, 99)}$=4.396, p<.001; Mean ± SEM, first session = 0.5 ± 0.15, last session = 1.46 ± 0.35], but did not differ across groups [Group, $F_{(2, 9)}$=1.387, p=.298; Session x Group, $F_{(22, 99)}$=1.262, p=.217]. For this reason and because the subsequent behavioural experiment used a completely within-subjects design, group was not included in subsequent analyses.

Habituation

After home-cage alcohol exposure, rats were left in their home-cages for 48 hours at which point they were brought to the behavioural testing room in their home-cages and individually handled.

Pavlovian Conditioning

The next day, Pavlovian conditioning was conducted to pair a discrete, auditory CS with alcohol delivery into a fluid port. All rats received 18 Pavlovian conditioning sessions (88.5 min duration) that began with a two-minute delay, after which the house-light was illuminated, and the first ITI began. In each session, 15 trials of an auditory CS (30 s; white noise) occurred on a VT 300 s schedule, with ITIs as the time between CS offset and the next CS onset of 180, 300, or 420 s. During CS presentations, 0.2 ml of alcohol was dispensed into the fluid port over 6 s (3 ml per session). Half of the Pavlovian conditioning sessions occurred in an *early* context wherein alcohol was delivered from the onset of the 5th second until the termination of the 10th second of each CS trial. The remaining conditioning sessions occurred in a *late* context, wherein alcohol was delivered from the onset of the 25th second until the termination of the 30th second of the same CS (**Fig 4a**). Sessions were alternated every day between the *early* and *late* contexts for each rat, according to a within-subjects design. Half of the rats began training in the *early* context and the remainder began training in the *late* context. Assignment to one of two different context types as the early or late context was random. Context type 1 included a smooth polycarbonate floor, lemon odour, and dark walls (black Bristol board outer cover). Context type 2 was a wire-mesh floor, almond odour, and clear walls. Context type and session order were counterbalanced.

Statistical Analyses

Software

Statistical analyses were conducted with SPSS^{TM} v20 and graphs were made with SigmaPlot^{TM} v12.

Dependent variables

During home-cage alcohol exposure, alcohol consumption as grams of ethanol consumed per kilograms of body weight (g/kg) were measured. In Pavlovian conditioning and test sessions the following responses were recorded, the number of: port entries per session (total port entries), port entries during the 10 s (Exp. 1) or 30 s (Exp. 2) CS (CS port entries),

port entries made between CS offset and the next CS onset (Exp. 1: 140, 260, or 380 s; Exp. 2: 180, 300, or 420 s; NonCS port entries), port entries in the 10 s (Exp. 1) or 30 s (Exp. 2) preceding the CS (PreCS port entries), and CS port entries minus PreCS port entries (Normalized CS port entries).

Analyses

Data from all experiments were analyzed with a repeated-measures ANOVA, and followed-up by post-hoc Bonferroni-corrected t-tests or simple main effects. When sphericity was violated Greenhouse-Geisser (GG) and Huynh-Feldt (HF) corrections are reported. All analyses used an alpha level of p=0.05.

Results

Experiment 1. The persistence of elevated CS responding in the alcohol context

Home-cage alcohol exposure

The dependent measure for home-cage alcohol exposure was grams of ethanol consumed as a function of rat weight in kilograms (g/kg). Alcohol consumption was analyzed using a Session (1, 2, 3…12) by Group (CS/NS, CS Only) repeated measures (RM) ANOVA. Alcohol consumption increased over 12 home-cage exposure sessions [Session, $F_{(11, 220)}$=3.875, p<.001] and did not differ for rats subsequently assigned to either the CS/NS or CS/NoNS groups [Group, $F_{(1, 20)}$<.001, p=.993; Session x Group, $F_{(11, 220)}$=1.106, p=.358; **Fig 1b**].

Experiment 1a. CS responding tests in the alcohol context and neutral context

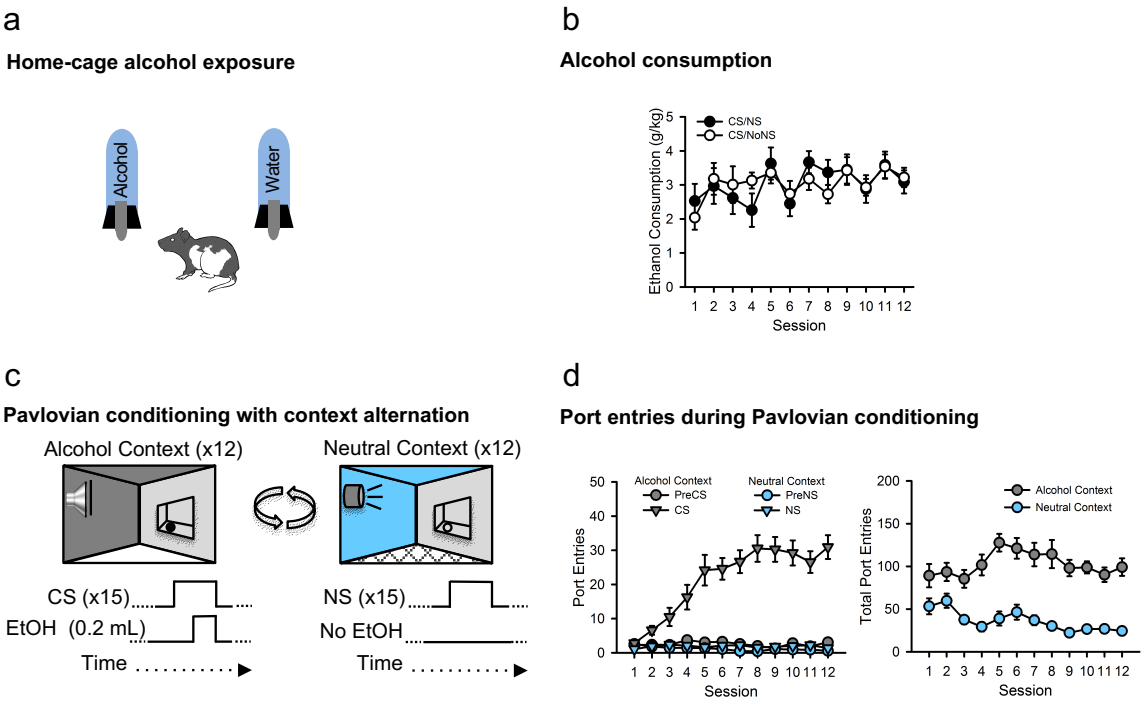
Two groups of rats underwent Pavlovian conditioning to pair a discrete CS with alcohol in a distinct alcohol context; ultimately there were no main effects or interactions with group and data is shown collapsed across this factor. On alternating days, the rats were placed into a different neutral context wherein alcohol was not available and one group of rats (CS/NS) was exposed to a neutral stimulus whereas the other group (CS/NoNS) received only controlled background noises. For the CS/NS group, port entries made during the PreCS and CS in the alcohol context were compared to port entries made during the PreNS and NS in the neutral context. For the CS/NoNS group, port entries made during the PreCS and CS in the alcohol context were compared to port entries made during two empty, consecutive, 10 s periods in the neutral context that corresponded to the PreCS and CS 260 s VT intervals. Thus, the factor interval refers to a similar period in the alcohol context for both groups, but for only the CS/NS group is there a stimulus, the NS, occurring during that period in the neutral context. Port entries

made across the acquisition of Pavlovian conditioning were analyzed using a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS or PreNoNS, CS/NS or NoNS) by Group (CS/NS, CS/NoNS) RM ANOVA. The total number of port entries made during each training session was analyzed using a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Group (CS/NS, CS/NoNS) RM ANOVA.

Port entries increased across sessions in the alcohol context relative to the neutral context [Session, $F_{(11, 220)}$=12.36, p<.001; Context $F_{(1, 20)}$=69.72, p<.001; Context x Session, $F_{(11, 220)}$=15.66, p<.001]. The elevation in port entries in the alcohol context was driven by CS port entries which increased into a plateau, whereas port entries during the PreCS remained stably low [Interval, $F_{(1, 20)}$=78.85, p<.001; Interval x Session, $F_{(11, 220)}$=18.58, p<.001]. Port entries during the NS, PreNS, NoNS and PreNoNS intervals in the neutral context all remained stably low throughout conditioning relative to CS port entries in the alcohol context [Context x Interval $F_{(1, 20)}$=66.67, p<.001]. The increase in CS relative to PreCS port entries in the alcohol context, while port entries during all intervals in the neutral context remained low and stable, suggests that rats specifically acquired responding to the CS in the alcohol context [Interval x Context x Session, $F_{(11, 220)}$=17.37, p<.001; **Fig 1d** (left)].

The acquisition of Pavlovian conditioning with context alternation was similar in both the CS/NS and CS/NoNS groups as evidenced by the lack of a four way Context x Interval x Session x Group interaction, all lower order interactions with Group, and the main effect of Group [Group, $F_{(1, 20)}$=.281, p=.602; Context x Group, $F_{(1, 20)}$=.017, p=.896; Interval x Group, $F_{(1, 20)}$=.793, p=.384; Session x Group $F_{(11, 220)}$=1.193, p=.293; Context x Interval x Group, $F_{(1, 20)}$=.127, p=.725; Context x Session x Group, $F_{(11, 220)}$=.738, p=.702; Interval x Session x Group, $F_{(11, 220)}$=.679, p=.758; Context x Interval x Session x Group, $F_{(11, 220)}$=.601, p=.827].

In the alcohol context, the total number of port entries was elevated relative to the neutral context [Context, $F_{(1, 20)}$=100.59, p<.001] and declined to a greater degree in the neutral context than in the alcohol context [Session, $F_{(11, 220)}$=2.48, p=.006; Session x Context, $F_{(11, 220)}$=3.45, p<.001; **Fig 1d** (right)]. Both the CS/NS and CS/NoNS groups showed a similar elevation in total port entries in the alcohol context relative to the neutral context and thus the main effect of Group and any interactions with Group were nonsignificant [Group, $F_{(1, 20)}$=.026, p=.873; Context x Group, $F_{(1, 20)}$=.001, p=.98; Session x Group $F_{(11, 220)}$=1.88, p=.044, .097 GG, .064 HF; Context x Group x Session, $F_{(11, 220)}$=1.93, p=.037, .08 GG, .047 HF].



Chapter 1 Figure 1: Experiment 1. The acquisition of Pavlovian conditioning with context alternation. **a** First, rats (n=22) received 24 h access to a 15% ethanol (alcohol) solution in their home-cage every other day for a total of 12 sessions. Water and standard rat chow were continuously available. **b** Alcohol consumption shown as grams of ethanol over kilograms of body weight across 12 home-cage alcohol exposure sessions. **c** Then, rats received 12 Pavlovian conditioning sessions every other day in a distinct alcohol context wherein a discrete, 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (12 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. While in the neutral context, half of the rats did not receive presentations of an NS (CS/NoNS group). The purpose of this groups was to determine if the acoustical salience of the neutral context would have an impact on CS port entries at test. Since there were no main effect or interactions as a function of group during acquisition or at test, the data reported here were collapsed across group. In all other experiments an NS was presented in the neutral context during training. **d** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral contexts across sessions of Pavlovian conditioning with context alternation (right). Averaged data are mean ± s.e.m.

Figure 1

After an equal number of training sessions in both contexts, responding to the CS was tested in the absence of alcohol, in both contexts on subsequent days that were intervened by retraining sessions (**Fig 2a**). A RM ANOVA was conducted for CS responding tests in the alcohol and neutral contexts that included the factors Context (Alcohol, Neutral), Interval (PreCS, CS) and Group (CS/NS, CS/NoNS). At test, rats made significantly more port entries in the alcohol context than in the neutral context [Context, $F_{(1, 20)}$=12.03, p=.002]. As well, more port entries were made during the CS than the PreCS [Interval, $F_{(1, 20)}$=127.68, p<.001], a difference in responding that was greater in the alcohol context than in the neutral context [Context x Interval, $F_{(1, 20)}$=14.66, p=.001; **Fig 2b** (left)]. A Bonferroni-corrected post-hoc t-test confirmed higher CS port entries at test in the alcohol context relative to the neutral context [$t_{(21)}$=3.736, p=.001]. At test, there was no overall difference in responding between the CS/NS and CS/NoNS groups as evidenced by the lack of a three-way Context x Interval x Group interaction, all lower order interactions with Group, and the main effect of Group [Group, $F_{(1, 20)}$=.297, p=.598; Interval x Group, $F_{(1, 20)}$=.173, p=.682; Context x Group, $F_{(1, 20)}$=.099, p=.756; Interval x Context x Group, $F_{(1, 20)}$=.229, p=.637].

At test, port entries made outside of the CS interval, NonCS port entries, may reflect general port-directed or unconditioned behaviour. NonCS port entries at test, were analyzed using a RM ANOVA with the factors Context (Alcohol, Neutral) and Group (CS/NS, CS/NoNS). At test, NonCS port entries were similar across contexts and groups [Context, $F_{(1, 20)}$=.363, p=.554; Group, $F_{(1, 20)}$=1.01, p=.327; Context x Group, $F_{(1, 20)}$=.122, p=.730; **Fig 2b** (right)].

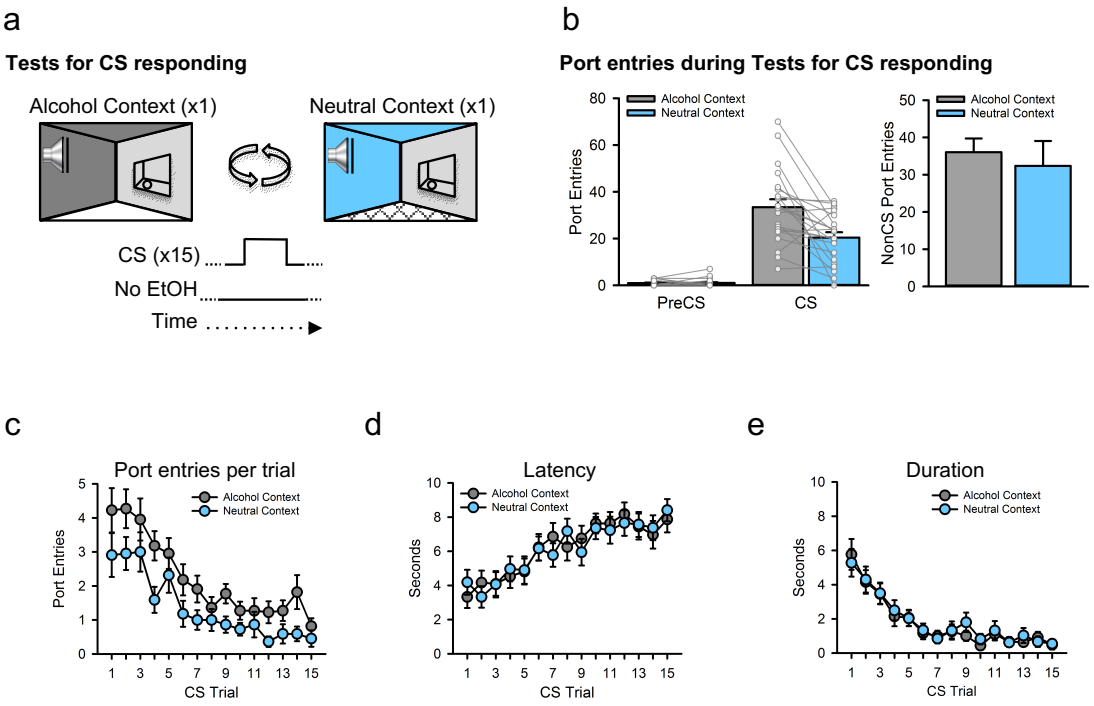
For test data, the average number of CS port entries (i.e. the total number of port entries made during each CS) per CS trial, the average latency to make a port entry after CS onset (i.e. the number of seconds after CS onset before the first port entry was made), and the average total duration of port entries initiated during the CS (i.e. the total time spent in the port during port entries initiated during the CS) were also measured. These three measures were analyzed in three separate RM ANOVAs with the factors Context (Alcohol, Neutral) and Trial (1…15) and Group (CS/NS, CS/NoNS).

At test the average number of CS port entries per trial decreased across CS trials similarly in both contexts [Trial, $F_{(14, 280)}$=16.243, p=.001; Trial x Context, $F_{(14, 280)}$=.530, p=.915; **Fig 2c**], but was elevated overall in the alcohol context [Context, $F_{(1, 20)}$=13.53, p<.001]. Both the CS/NS and CS/NoNS groups showed similar trajectories of CS responding across trials which is

reflected in the lack of a significant effect of group and all interactions with group [Group, $F_{(1, 20)}$=.219, p=.644; Context x Group, $F_{(1, 20)}$=.147, p=.706; Trial x Group, $F_{(14, 280)}$=1.439, p=.135; Context x Trial x Group, $F_{(14, 280)}$=.910, p=.549].

The latency to initiate a CS port entry after CS onset increased as trials progressed [Trial, $F_{(14, 280)}$=12.329, p<.001] and neither the trajectory nor the latency differed significantly across contexts [Context, $F_{(1, 20)}$=.006, p=.939; Context x Trial, $F_{(14, 280)}$=.488, p=.939; **Fig 2d**]. Both the CS/NS and CS/NoNS groups showed similar latencies to respond which is reflected in the lack of a significant effect of group and all interactions with group [Group, $F_{(1, 20)}$=.853, p=.367; Context x Group, $F_{(1, 20)}$=.075, p=.789; Trial x Group, $F_{(14, 280)}$=1.247, p=.241; Context x Trial x Group, $F_{(14, 280)}$=.493, p=.936].

The average duration of CS port entries at test decreased along a similar trajectory across trials in both contexts [Trial, $F_{(14, 280)}$=19.79, p<.001; Context, $F_{(1, 20)}$=.169, p=.685; Context x Trial, $F_{(14, 280)}$=.279, p=.996; **Fig 2e**]. The CS/NS and CS/NoNS groups did not differ in terms of the average duration of CS port entries at test as the main effect of group and all higher order interactions with group were nonsignificant [Group, $F_{(1, 20)}$=.228, p=.638; Context x Group, $F_{(1, 20)}$=.835, p=.372; Trial x Group, $F_{(14, 280)}$=.926, p=.531; Context x Trial x Group, $F_{(14, 280)}$=.695, p=.779].



Chapter 1 Figure 2: Experiment 1. CS responding tests in the alcohol and neutral context. **a** After Pavlovian conditioning with context alternation rats (n=22; same as figure 1) received tests for responding to the discrete alcohol conditioned stimulus (CS). During tests, the CS was presented (15 trials per session; variable inter trial interval = 260 s) in the alcohol and neutral context on separate days without alcohol and tests were intervened by retraining sessions. **b** Port entries made during the PreCS and CS intervals during tests (left). Port entries made in between CS trials (NonCS) at test in the alcohol and neutral context (right). **c** The average number of CS port entries (i.e. the total number of port-entries made during each CS) across CS trials during test sessions. **d** The average latency to make a port entry after CS onset (i.e. the number of seconds after CS onset before the first port-entry was made) across CS trials during test sessions. **e** The average total duration of port-entries initiated during the CS (i.e. the total time spent in the port during port-entries initiated during the CS) across CS trials during test sessions. Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 2

Experiment 1b. Repeated test sessions in the alcohol context and neutral context

After CS responding was tested in both contexts, eight additional tests were conducted (four per context) to examine the persistence of CS responding in the alcohol and neutral context (**Fig 3a**). For clarity, data from the repeated test sessions (Exp. 1b) and the reinstatement test (Exp. 1c) are shown as normalized CS port entries, calculated by subtracting PreCS port entries from port entries during the corresponding CS. This subtraction accounts for individual differences in baseline levels of port entry behaviour. Normalized CS port entries made across repeated test sessions were analyzed using a Session (1, 2, 3, 4) by Context (Alcohol, Neutral) RM ANOVA.

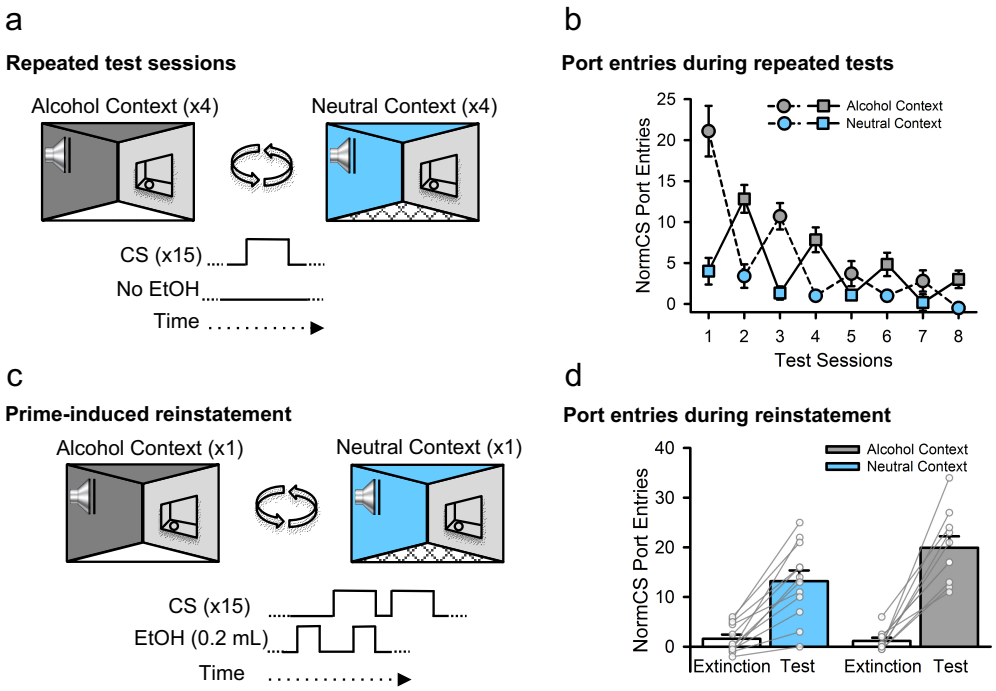
For the analyses of experiment 1a group was included as a factor but the two groups (CS/NS, CS/NoNS) are shown cumulatively in the same graphs because the main effect of group, and any interaction with group, were nonsignificant. For the repeated test session (Exp. 1b) and reinstatement (Exp. 1c) phases, the CS/NS, and CS/NoNS groups are treated as a single group on the basis of the lack of any significant group effects in previous phases, and because they are treated similarly in experiments 1b and c. In other words, during the repeated test sessions and the reinstatement test all rats received only the CS in each context, and the previous CS/NS, and CS/NoNS affiliation became irrelevant.

During repeated test sessions, normalized CS port entries were higher in the alcohol context than the neutral context [Context, $F_{(1, 10)}$=30.671, p<.001] but waned across sessions [Session, $F_{(3, 30)}$=18.435, p<.001]. Normalized CS port entries were maintained for more sessions in the alcohol context than in the neutral context wherein port entries dropped to low levels rapidly [Context x Session, $F_{(3, 30)}$=5.885, p=.003; **Fig 3b**].

Experiment 1c. Reinstatement in the alcohol context or neutral context

After responding had extinguished to similarly low levels in both contexts, a reinstatement test was conducted by delivering a drop of alcohol to serve as a reminder of the orosensory properties of alcohol (**Fig 3c**). Normalized CS port entries made during the last two repeated test sessions (one in each context) were averaged to produce a baseline against which responding during the reinstatement test could be compared. Normalized CS port entries made during the reinstatement test were analyzed using a Context (Alcohol, Neutral) by Phase (Extinction Baseline, Reinstatement) between-subjects repeated measures ANOVA. One group of rats received a reinstatement test in the alcohol context (n=10) and the other in the neutral

context (n=12). The main effect of Reinstatement context was not significant [Reinstatement Context, $F_{(1, 20)}$=2.786, p=.111]. Relative to an averaged baseline of the previous two extinction sessions, normalized CS port entries were reinstated in both contexts [Phase, $F_{(1,20)}$=108.159, p<.001], but to a greater extent in the alcohol context than in the neutral context [Phase x Reinstatement Context, $F_{(1, 20)}$=6.037, p=.023; **Fig 3d**]. A Bonferroni-corrected post-hoc t-test indicated higher normalized CS port entries at test in the alcohol context relative to the neutral context [$t_{(20)}$=2.112, p=.047].



Chapter 1 Figure 3: Experiment 1b. Repeated test sessions and reinstatement in the alcohol and neutral context. **a** After the last test for CS responding (figure 2), rats (n=22; same as figure 1) received the first of 8 (4 in each context) consecutive additional test session. Repeated test sessions were conducted by presenting the CS without alcohol in the alcohol and neutral context every other day. **b** Normalized CS (NormCS; i.e., CS minus PreCS) port entries across repeated test sessions. Half of the rats received their first additional test in the alcohol context (dashed line) whereas the remainder received their first additional test in the neutral context (solid line). **c** After the last repeated test session, rats received prime-induced reinstatement tests in which a drop of alcohol (0.2 ml) was presented in the fluid port 90 s before and during the first CS presentation. Half of the rats received the reinstatement test in the neutral context following their last extinction session in the alcohol context and vice versa for the remainder. **d** Normalized CS port entries in the alcohol and neutral context during reinstatement tests and an extinction baseline which reflects averaged normalized CS port entries across the last two extinction sessions. Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 3

Experiment 2. The capacity for context to control the timing of CS responses

This experiment was conducted to test the hypothesis that, in addition to increasing the magnitude of responding as reported in experiment 1, context could control the timing of responses to a discrete alcohol CS. The probability of a port entry occurring during the CS (30 s) or PreCS (30 s before CS) intervals, or during specific time bins within the CS, was calculated by summing the number of trials within a session in which at least one port entry occurred and dividing the sum by the total number of trials[120]. Acquisition was analyzed using a Session (1-9) by Context (Early, Late) by Interval (PreCS, CS) RM ANOVA. The probability of a port entry occurring during the first 4 s of the CS or during the 6 s of alcohol delivery was analyzed using separate RM ANOVAs with the factors Context (Early, Late) and Session (1, 5 and 9 to represent the beginning, middle, and end of conditioning).

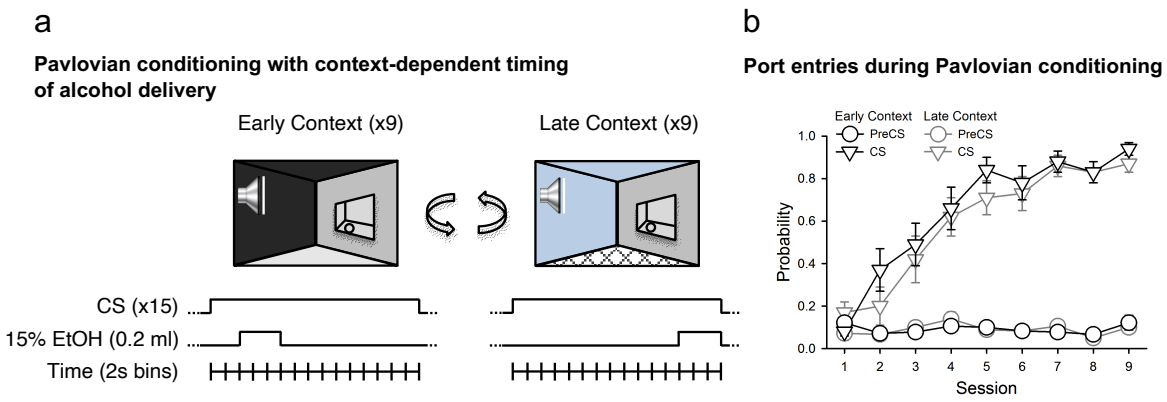
Across 18 sessions of conditioning (nine in each context; **Fig 4a**), port entry probability was higher during the CS than the PreCS [Interval, $F_{(1, 11)}$=132.722, p<.001] and increased across sessions [Session, $F_{(8, 88)}$=26.018, p<.001] during the CS [Interval x Session, $F_{(8, 88)}$=34.009, p<.001]. Responding did not differ between contexts across sessions [Context x Session, $F_{(8, 88)}$=.870, p=.545]. However, overall response probability was higher in the early context than the late context [Context, $F_{(1, 11)=}$4.87, p=.049]. This difference was driven by a higher probability of CS port entries in the early context than the late context [Context x interval $F_{(1, 11)=}$8.818, p=.013], which showed a trend towards changing across session [Context x Interval x Session, $F_{(8, 88)}$=2.002, p=.055; **Fig 4b**].

To further explore the near-significant 3-way interaction of Context x Interval x Session, a targeted analysis of CS port entries in sessions 1, 5 & 9, which represented the beginning, middle and end of acquisition was conducted. Bonferroni-corrected t-tests revealed that the probability of CS port entries was not different across contexts in session 1 [$t_{(11)}$=-1.497, p=.163] or session 9 [$t_{(11)}$=-1.593, p=.140], but was higher in the early context in session 5 [$t_{(11)}$=4.454, p=.001]. Thus, the acquisition of CS port entries was more rapid in the early context than the late context, which is to be expected given the shorter interval between the CS and US in the early context[112]. However, by the end of acquisition, response probability during the CS did not differ as a function of context.

The first four seconds of each CS trial represented a similar interval in both contexts, in that CS onset had occurred but US delivery had not (**Fig 5a**). An analysis of the influence of

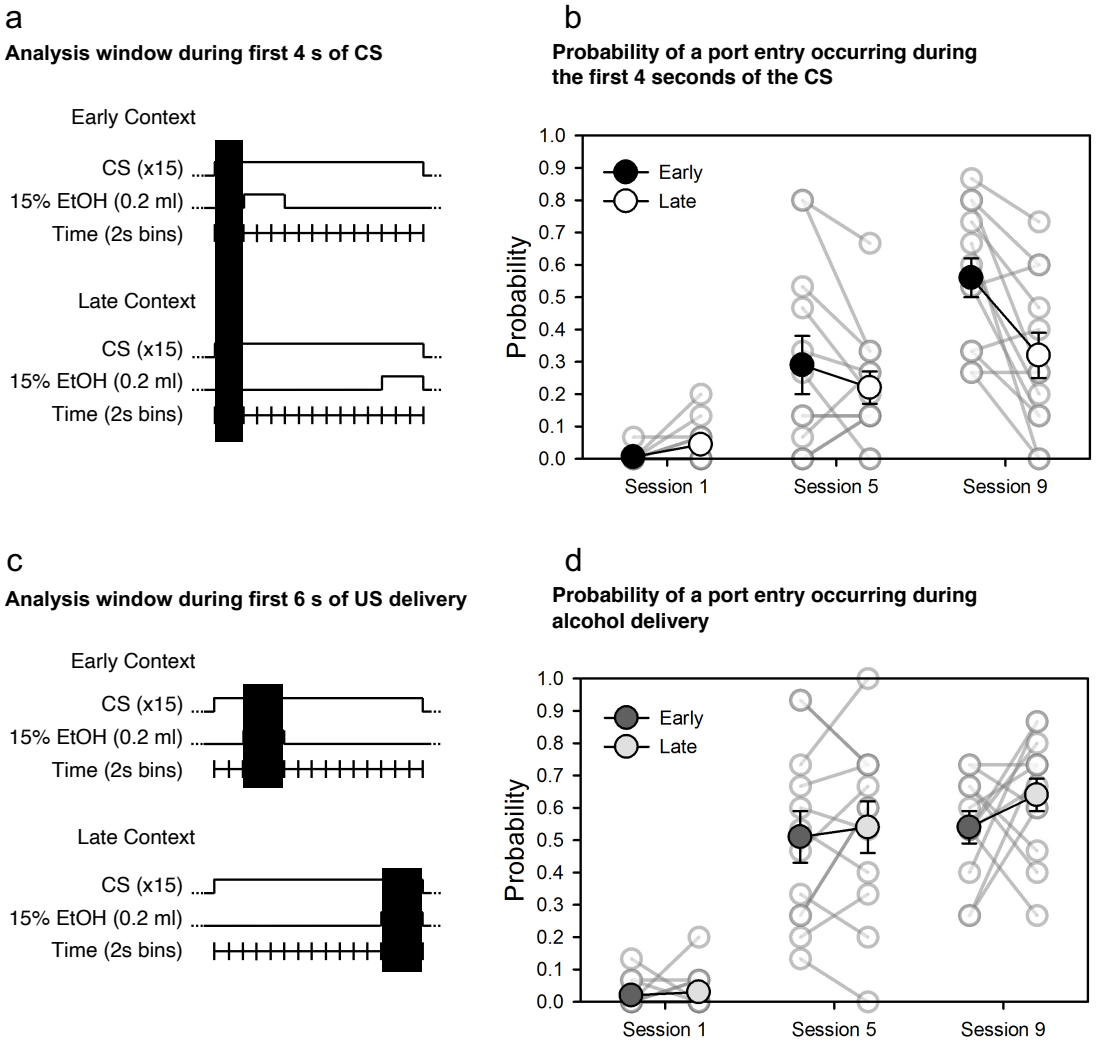
context on responding during this interval in sessions 1, 5 and 9, which represented the beginning, middle and end of acquisition was conducted. The probability of a CS port entry occurring during the first four seconds was higher in the early context than the late context [Context, $F_{(1, 11)}$=10.260, p=.008], and increased across sessions 1, 5 and 9 [Session, $F_{(2, 22)}$=28.893, p<.001] to a greater extent in the early context than in the late context [Session x Context, $F_{(2, 22)}$=7.532, p=.003; **Fig 5b**]. Follow-up Bonferroni-corrected t-tests indicated that the probability of a port entry occurring during the first four seconds of the CS was similar across contexts in sessions 1 and 5 (**Fig 5b**; p>.2), but was significantly higher in the early context than the late context in session 9 [**Fig 5b**; $t_{(11)}$=3.684, p=.012]. Thus, in a session in which the overall probability of responding during the CS did not differ, the probability of responding in the first four seconds of the CS was higher in the early context than the late context.

Port entries during alcohol delivery in sessions 1, 5 and 9 (**Fig 5c**) were analyzed to confirm that differences in responding during the first four seconds of the CS did not reflect baseline differences in port entry responding. The probability of a port entry occurring during the US increased across sessions 1, 5 and 9 [Session, $F_{(2, 22)}$=82.807, p<.001], but did not differ as a function of context [Context x Session, $F_{(2, 22)}$=.475, p=.628; Context, $F_{(1, 11)}$=.954, p=.350; **Fig 5d**].



Chapter 1 Figure 4: Experiment 2. The capacity for context to control the timing of responses to a discrete CS. **a** Rats (n=12) received 18 Pavlovian conditioning sessions in which a 30 s discrete auditory CS (15 trials per session; variable inter trial interval = 280 s) was paired with alcohol delivery (0.2 ml per trial; 3 ml per session) into a fluid port for oral consumption. Conditioning sessions were conducted in two distinct contexts (9 sessions each) on alternating days using a within-subjects design. In one context, called the early context, alcohol was delivered from the onset of the 5th second until the termination of the 10th second of a 30 s CS. In the second, late context, alcohol was delivered from the onset of the 25th second until the termination of the 30th second of the same CS. **b** The probability of a port entry occurring during the CS and the PreCS intervals in the early and late contexts. All averaged data are shown as mean ± s.e.m.

Figure 4



Chapter 1 Figure 5: Experiment 2. The capacity for context to control the timing of responses to a discrete CS. **a** A schematic showing the 4 second analysis window (grey box) relative to CS onset and alcohol delivery in the early and late contexts. **b** The mean ± s.e.m. probability of a port entry occurring during the first four seconds of the CS in the early (black circles) and late (white circles with black outline) contexts in sessions 1, 5, and 9. **c** A schematic showing the 6 second analysis window (grey box) relative to CS onset and alcohol delivery in the early and late contexts. **d** The mean ± s.e.m. probability of a port entry occurring during the six seconds of alcohol delivery in the early (dark grey circles) and late (light grey circles) contexts in sessions 1, 5, and 9. In **b** and **d**, grey transparent circles without error bars represent individual data.

Figure 5

Discussion

The capacity for environmental cues to engender alcohol-seeking behaviour is recognized as a difficult problem to address in alcohol use disorder recovery[20]. A critical step in understanding this problem is to isolate the types of environmental cues that can acquire incentive motivational properties and describe the extent to which their combined and independent presentation affects behaviour. In experiment 1, it was shown that an alcohol context elevated responding to a discrete alcohol CS, and that experiencing an acoustically impoverished neutral context during training did not undermine previous reports[57,73] of this effect. Experiments 1b and c furthered the finding that CS responding was elevated at test in the alcohol context by showing that this effect persisted across multiple repeated test sessions and that CS responding was reinstated to a higher level in the alcohol context. While experiment 1 confirmed that context influenced the magnitude of CS responding, experiment 2 extended this finding by showing that context can also control the timing of responses elicited by a discrete alcohol CS. Together, these behavioural findings highlight the robustness, persistence, and temporal sensitivity with which an alcohol context can influence responding to a discrete alcohol CS.

An important finding from the current set of studies is that the capacity for an alcohol context to elevate CS port entries cannot be explained by having an acoustically impoverished neutral context during training. In previous studies using Pavlovian conditioning with context alternation[57,73], the neutral context was devoid of a discrete auditory stimulus during training, and the CS responding test was the first session in which a discrete auditory stimulus was presented in this context. It has been demonstrated previously that the volume of static background noise and the frequency of discrete stimulus presentations can sensitize or habituate a startle response to a discrete auditory stimulus[107], which informed the concern about acoustic salience in the current procedure. The lack of a discrete auditory stimulus in the neutral context may have contributed to responding at test when the CS is presented in this context for the first time. Experiment 1a, demonstrated that equating the acoustic salience of the alcohol and neutral contexts by including a NS in the neutral context during training does not impact responding during any interval in training and test sessions. Therefore, the capacity for the alcohol context to elevate CS responding, as demonstrated in the current and previous studies[57,73], cannot be explained by an acoustically impoverished neutral context during training.

Experiments 1b and c demonstrated that the capacity for an alcohol context to elevate CS responding persisted across multiple repeated test sessions and was retained after extinction. This finding, that the influence of context over responding to a discrete alcohol CS was long-lasting, has implications for alcohol use disorder in people. For example, a goal of cue-exposure therapy is to extinguish the reactivity that people experience when presented with alcohol cues[19,20]. The elevation of CS responding in the alcohol context relative to the neutral context during repeated test sessions suggests that aiming to extinguish cue-reactivity outside of real-world drinking contexts in people overestimates the efficacy of extinction. Furthermore, exposure to an oral alcohol prime after extinction reinstated CS responding in both contexts, but to a greater level in the alcohol context. The oral alcohol prime had not been extinguished previously and thus was an effective trigger for relapse in the prime-induced reinstatement procedure. This result suggests that people that have undergone cue-exposure therapy to certain cues remain vulnerable to relapse in the presence of non-extinguished cues, particularly when those cues are presented in an alcohol context. Together, the findings from experiments 1b and c suggest that the efficacy of cue-exposure therapy conducted outside of real-world drinking contexts is overestimated and further that people remain vulnerable to relapse when presented with non-extinguished alcohol cues.

In addition to demonstrating the capacity for context to persistently elevate CS responding, it was shown that context can also control the timing of responses elicited by a discrete alcohol CS. In experiment 2, a modified version of the Pavlovian conditioning procedure used in experiment 1 was developed to examine whether context could control the timing of CS responses for alcohol. Notably, in a context in which alcohol was delivered earlier with respect to CS onset, rats exhibited a higher probability of anticipatory responding during the CS when compared to a different context in which alcohol was delivered later during the same CS. Interestingly, during the CS responding test in experiment 1a, CS port entries were elevated in the alcohol context relative to the neutral context, but the latency of CS port entries did not differ across context. This finding may suggest that context influences the magnitude of responding without affecting other features of the response form. While this suggestion appears true in experiment 1a, it was shown in experiment 2 that when context signals the onset of alcohol delivery during a discrete CS, context also guides the timing of responding during the CS. Thus, while context influenced only the magnitude of responding in experiment 1, experiment 2 shows that context has the capacity to control the timing of CS responses, which is demonstrable in appropriately designed conditioning procedures.

In chapter 1, two Pavlovian conditioning procedures were used to describe the influence of context over responding to a discrete alcohol CS. Experiment 1 replicated the finding that an alcohol context elevated responding to a discrete alcohol CS relative to a neutral context[57,73], and showed that this effect persisted across repeated test sessions and re-emerged in a reinstatement test. Importantly, by including a group that received a NS in their neutral context during training the potential caveat that having an acoustically impoverished neutral context could affect behaviour at test, was addressed. Further, it was demonstrated that the lack of an effect of context on the latency to respond during the CS at test in experiment 1a should not be taken as evidence that context lacks the capacity to influence the timing of CS responses. In experiment 2, using an appropriately designed conditioning procedure, it was shown that context can control the timing of responses to a discrete alcohol CS. In summary, chapter 1 demonstrated that an alcohol context persistently elevated responding to a discrete alcohol CS and has the capacity to control the timing of CS responses.

Chapter 2: The necessity of separable VTA dopamine circuits for responding to a discrete alcohol CS in different contexts

Introduction

The process by which food and drug conditioned stimuli are imparted with incentive motivational properties is dopamine-dependent[121,122] and thought to underlie problematic responses to CSs that occur in lab animals and people[123–125]. In terms of alcohol, both discrete cues[126] and contexts[79] associated with alcohol evoke dopamine release in the nucleus accumbens which is densely innervated by dopaminergic neurons originating in the ventral tegmental area (VTA)[58,67,68,127]. Further, the NAc core and shell subregions are hypothesized to have dissociable roles in behaviour that is motivated by discrete cues and contexts[56,95,128]. Chapter 2 had two main aims. First, the hypothesis that VTA dopamine activity is necessary for responding to a discrete alcohol cue was examined. Second, the hypothesis that the dopaminergic projections from the VTA to the NAc core and shell disparately support responding to a discrete alcohol cue, and the elevation of this behaviour in an alcohol context, respectively, was investigated.

The dopamine system has been implicated in the context renewal model of alcohol relapse[53,71,72]. Often renewal procedures involve tests for responding in two contexts: one wherein alcohol had been paired with a discrete cue and another wherein the discrete cue-alcohol association was extinguished. Some Pavlovian renewal studies indicate that D1-like dopamine antagonists reduce responding for alcohol in an alcohol-associated context but not in an extinction context wherein alcohol was never available[72]. However, other studies show that both D1-like and D2-like antagonists reduce Pavlovian responding for alcohol in a neutral context where alcohol was never available[106]. The discrepant results from these studies might arise from differences in overall exposure to the neutral and extinction contexts relative to the alcohol context, the lack of extinction training in the neutral context, or the blocking of context exposures into distinct phases. It is unclear, whether dopamine neurotransmission at D1- and D2-like receptors is required for responding to a discrete Pavlovian CS in a neutral context that is equally familiar to an alcohol context wherein Pavlovian conditioning occurred. Experiment 1 was conducted to test whether dopamine D1-like (SCH23390) and D2-like (Eticlopride) dopamine receptor antagonists impacted responding to a discrete alcohol CS in a neutral context.

The results from experiment 1, suggested that the dopamine system was necessary for responding to a discrete alcohol CS, however systemic pharmacology does not permit parsing of dopaminergic neurons originating from different parts of the midbrain[68] which are thought to underlie separable aspects of alcohol-seeking behaviour[129]. A chemogenetic[130] approach allows for the manipulation of neural activity to be confined to a specific brain area, offering a further level of anatomical specificity compared to systemic pharmacology. Specifically, viral constructs can be microinfused into defined brain areas, confining the actions of inhibitory or excitatory designer receptors which respond to the ligand clozapine-*n*-oxide (CNO)[130,131]. An additional level of specificity can be achieved by combining double-inverted open reading frame (DIO) viral constructs[132] with transgenic animals that express the enzyme cyclic recombinase (Cre) in a defined cellular population, because DIO constructs require the presence of Cre to be appropriately transcribed and translated into proteins. The transgenic TH::Cre rat[133] expresses Cre in cells that produce tyrosine hydroxylase (TH), an enzyme necessary for the synthesis of dopamine and other catecholamines. As such, chemogenetics can be used in TH::Cre rats to target dopamine neurons in specific brain areas without directly affecting other neurotransmitter systems.

To refine the anatomical specificity of the results from experiment 1, which implicated the dopamine system in responding to a discrete alcohol CS, experiment 2 used chemogenetics in TH::Cre rats to test the necessity of VTA dopamine neurons for responding to a discrete alcohol CS in a neutral context. Specifically, TH::Cre rats received microinfusions of a DIO viral vector encoding the inhibitory designer receptor (hM4Di), allowing the inhibitory designer receptor to be selectively expressed in VTA dopamine neurons. Before tests for CS responding, rats received systemic injections of CNO to inhibit the activity of VTA dopamine neurons[134]. CNO was thought to have a selective effect on designer receptors[130], however recent reports have suggested that CNO can be metabolized to its parent compound clozapine, which is an atypical antipsychotic[135], and produce off-target behavioural effects[136]. Although, the suggestion that CNO may be a problematic ligand for designer receptor has met backlash[137–139], it is prudent to rule out competing explanations for behavioural chemogenetic effects by administering CNO and clozapine in the absence of designer receptors. Experiment 3 was conducted to test for potential off-target effects of CNO and its parent compound clozapine on responding to a discrete alcohol CS in TH::Cre rats that expressed only a control fluorescent protein (mCherry) in VTA dopamine neurons. Together, experiments 2 and 3 were designed to test the necessity

of VTA dopamine neurons for responding to a discrete alcohol CS while assessing the potential off-target effects of CNO and its parent compound clozapine.

Observational and perturbational studies support the hypothesis that VTA dopamine neurons are involved in the influence of context over responses that are elicited by discrete cues. For example, the activity of VTA dopamine neurons in response to a discrete CS that predicts an aversive stimulus is modulated by the context in which that stimulus is presented[103]. Further, context renewal of operant responding for alcohol is attenuated by the administration of dopamine antagonists into the NAc core and shell[71]. Interestingly, responding to an alcohol CS in a neutral context, requires activity in the NAc core, but not shell, which suggests that these two subregions are disparately involved in responding to discrete alcohol cues, and contexts[56]. Additional support for this disparity comes from a study showing that antagonism of dopamine D1-like receptors in the NAc shell, but not core, reduced context-induced renewal of heroin-seeking whereas the same manipulation in the NAc core, but not shell, reduced responding that was reinforced by a discrete heroin cue[95]. The separable involvement of the NAc core and shell in behaviours motivated by discrete cues and contexts, and the modulation of VTA activity by context, suggests that dopaminergic projections from the VTA to the NAc core and shell might subserve responding to discrete cues and the modulation of this behaviour by context, respectively.

There is considerable evidence to support the hypothesis that activity in the NAc core and shell underpin separable aspects of responding for alcohol. However, extending this hypothesis to ascribe specific roles for dopaminergic VTA-to-NAc core and shell projections, requires that at least two caveats are overlooked. First, operant responding can be supported by the intrinsically reinforcing properties of non-drug stimuli, such as flashes of light[35–37]. It is unclear from studies showing a role for the NAc core in responding for discrete heroin cues whether responses were motivated by the reinforcing properties intrinsic to the discrete stimuli used, or the acquired reinforcing properties of drug-paired stimuli. Second, responding in operant renewal procedures that is thought to be motivated by discrete cues or contexts, is often confounded by the presentation of discrete cues in a response-contingent manner and context in a response-independent manner. Thus, the dissociation in NAc core versus shell function suggested by renewal experiments could be related to the active (response-contingent) versus passive (response-independent) nature of discrete cue and context presentation[95]. These two caveats need to be considered when interpreting the effects of neurobiological

manipulations on behaviour in the renewal procedure, and other procedures involving conditioned responding for cues.

Pavlovian models of renewal circumvent the confounding of active and passive discrete cue and context presentation. However, it can be difficult to attribute behaviour to discrete cues or contexts in the renewal procedure. Often renewal procedures block training into phases of acquisition and extinction in separate contexts, before testing responding in both the acquisition and extinction contexts, or in a novel context[105]. This blocking of context exposures produces an inequity in the frequency and overall number of exposures to test contexts. Further, the nature of what is learned in the extinction and acquisition contexts, is vastly different. For example, extinction learning becomes embedded in the context in which extinction occurs[140,141] and this type of learning is absent in the acquisition context. It is difficult to conclude that responding in the extinction or acquisition context is motivated purely by the discrete cue, or the concomitant presentation of the discrete cue and context.

Experiments 4 and 5 were designed to delineate the contributions of the dopaminergic projections from the VTA to the NAc core and shell in responding to a discrete alcohol cue in different contexts. The Pavlovian conditioning with context alternation procedure allowed for comparisons across contexts that are equally familiar but differ in their association with alcohol, which was critical to test the proposed hypotheses of separable VTA-to-NAc core and shell function. To target VTA-to-NAc projections, a circuit-specific chemogenetic approach was used by microinfusing a DIO viral construct encoding for the inhibitory designer receptor in the VTA of TH::Cre rats, and implanting cannulae targeting either the NAc core (Exp. 4) or shell (Exp. 5). Cannulae, were used to deliver CNO into the target region to selectively inhibit the dopaminergic projection from the VTA to the NAc core or shell, before tests for CS responding in the alcohol or a neutral context. If the dopaminergic VTA-to-NAc core projection was necessary for responding to a discrete alcohol CS, then inhibiting this projection should reduce CS responding irrespective of context. In contrast, if the VTA-to-NAc shell projection was necessary for the elevation of CS responding in the alcohol context, then inhibiting this projection should reduce responding only in the alcohol context.

An important feature of experiments 4 and 5, was the use of chemogenetics in TH::Cre rats to target and manipulate the activity of VTA dopamine projections to the NAc core and shell. It had been demonstrated previously in TH::Cre rats, that designer receptors could be

selectively expressed in TH positive VTA neurons and that these receptors were trafficked to terminals in the NAc[134,142–145]. This selective expression of designer receptors on TH positive VTA neurons was reaffirmed here using immunocytochemistry (Exp. 6). In addition to the anatomical validation of the chemogenetic approach, a functional validation was required. Researchers had shown previously using c-fos[134] and electrophysiology[134,143] that designer receptors expressed on VTA dopamine neurons could impact activity at the level of the cell body. However, only one study had demonstrated that inhibitory and excitatory designer receptors expressed on VTA dopamine terminals in the NAc could modulate evoked dopamine release[134], and no such study had determined whether this modulation affected the activity of postsynaptic medium spiny neurons (MSNs). Thus, a critical validation for the current set of experiments was to determine whether inhibitory designer receptors expressed on VTA dopamine terminals in the NAc could affect the activity of postsynaptic MSNs. To this end, in experiment 7, electrically-evoked excitatory postsynaptic potentials were recorded from MSNs in the NAc core that were innervated by VTA dopamine terminals expressing inhibitory designer receptors. Experiments 6 and 7 provided an important anatomical and functional validation of the circuit-specific chemogenetic approach used in the current thesis.

In chapter 2, first, pharmacology (Exp. 1) and chemogenetics (Exps. 2 & 3) were used to test whether VTA dopaminergic activity was necessary for responding to a discrete alcohol CS. Next, a circuit-specific chemogenetic approach was used to test the prediction that the dopaminergic VTA-to-NAc core projection was necessary for responding to a discrete alcohol CS (Exp. 4), whereas the VTA-to-NAc shell projection was necessary for the elevation of this behaviour in an alcohol context (Exp. 5). Lastly, anatomical (Exp. 6) and functional (Exp. 7) validations of the chemogenetic approach in TH::Cre rats were performed.

Methods

Subjects

Male, Long-Evans rats (n=11; CS/NS group from Ch 1 Exp. 1) were ordered from ENVIGO Laboratories, Indianapolis, USA and weighed 220-275 g on arrival (Exp. 1). Male, Long-Evans, TH::Cre$^{+/-}$ rats (n=44) were bred in-house on a mixed Charles River and INVIGO background and used in experiments 2 (n=12), 3 (n=13), 4 (n=8), 5 (n=11), 6 (n=4), and 7 (n=5). All rats were individually housed in standard polycarbonate shoebox cages (20 x 24 x 45 cm) and maintained on a 12 h light-dark cycle (lights on at 0700) at 21±2°C at 40-50% humidity. All procedures were conducted in the light phase and rats had unrestricted access to chow

(Charles River Rodent Diet #5075), tap water, and a nylabone™ chew-toy. All experimental procedures complied with the Animal Research Ethics Committee at Concordia University and the Canadian Council on Animal Care regulations.

In total, 11 rats failed to acquire Pavlovian conditioning (mean of <5 CS port entries/ session across last two training sessions), 6 had missed cannula placements, and 1 had the house-light burn out during a test. Data from these rats were excluded.

Behavioral apparatus

Behavioral training and testing occurred in 12 conditioning chambers (ENV-009A; Med-Associates Inc.), enclosed in fan-ventilated (~77 dB), sound-attenuating, melamine cubicles (53.6 x 68.2 x 62.8 cm). The right wall featured a fluid port (17.5 cm from rear wall, 9 cm from front door) that contained two wells (ENV-200R3AM). Fluid delivery into one well occurred through a 20 ml syringe attached to a pump (PHM-100, 3.33 rpm) located outside the cubicle. Fluid port entries were measured with an infrared beam (ENV-205M) and recorded to a computer using Med PC-IV software, which also controlled fluid delivery and stimulus presentations. The upper left wall featured a clicker stimulus (ENV-135M, 8 dB above background), a continuous white noise stimulus generator (ENV-225SM, 8 dB above background), and a white house-light (ENV-215M).

Solutions and reagents

Odours were prepared by adding lemon oil (SAFC Supply Solutions, St-Louis, MO, USA, #W262528) or benzaldehyde (almond odor, OMEGA Chemical Company Inc., Levis, QC, Canada, #B37-50) to tap water (10%, v/v). Alcohol (15 % ethanol, v/v) was prepared every week by diluting 95% ethanol in tap water (room temperature). Eticlopride ($C_{17}H_{25}ClN_2O_3 \cdot HCl$, Sigma Aldrich, #E101) and SCH23390 ($C_{17}H_{18}ClNO \cdot HCl$, Sigma Aldrich, #D054) were dissolved in sterile 0.9% saline to make separate 10 μg/ml solutions. CNO (Clozapine-*n*-oxide, Tocris #4936 or NIMH C-929) for systemic administration was dissolved in 5% dimethyl sulfoxide (DMSO) and 95% sterile 0.9% saline to make 10 or 20 mg/ml concentrations. Clozapine (AdooQ, #A10236-500) was dissolved in 5% dimethyl sulfoxide and 95% sterile 0.9% saline to make a 0.1 mg/ml solution. CNO (abcam, #ab141704) was dissolved in sterile 0.9% saline (3 mM for intracerebral microinfusions) or artificial cerebrospinal fluid (1 μM for *in vitro* electrophysiology). Viral vectors were bought from the University of North Carolina Vector Core [AAV8-hSyn-DIO-hM4D(Gi)-mCherry (titer 5.3 or $4.6x10^{12}$), AAV8-hSyn-DIO-hM3D(Gq)-

mCherry (titer 5.9x10^{12})] or Addgene [AAV8-hSyn-DIO-hM4D(Gi)-mCherry (titer 2.06x10^{12}), AAV8-hSyn-DIO-mCherry (titer 2.1x10^{13})].

Surgery

Anesthetized (isoflurane, 5% induction, 2-3% maintenance) rats were secured in a stereotaxic frame and administered atropine (0.1 ml/kg) subcutaneously (s.c.). Bilateral, ventral tegmental area (VTA) microinfusions of 1 μl (0.1 μl/min, 10 min diffusion) of AAV8-hSyn-DIO-hM4D(Gi)-mCherry, AAV8-hSyn-DIO-mCherry, or AAV8-hSyn-DIO-hM3D(Gq)-mCherry viral vectors were made through a 26 gauge injector connected with PE20 tubing to a Hamilton microinjection syringe on a Harvard Apparatus, Pump 11 Elite. VTA coordinates (in mm) from bregma were: AP -5.5, ML ±1.84 (with 10° angle), DV -8.33.

Where appropriate, 26-gauge bilateral guide cannulae (PlasticsOne, C315G-SPC) were implanted 3 mm dorsal to the microinjection site using the following coordinates (in mm) at a 10° angle: NAc core AP +1.2, ML ± 3.23, DV -7.11, and NAc shell AP +1.68, ML ± 2.23, -7.35. After surgery, rats received buprenorphine (0.1 mg/kg, s.c.) and ≥7 days to recover.

General behavioural procedures

Home-cage alcohol exposure

Imposing intermittent schedules of alcohol availability has been shown to produce gradual elevations in ethanol consumption[106,114–119]. All rats received 24 h access to alcohol and tap water every other day for 12 sessions, over 24 days. On the intervening days only, water was available. Alcohol was provided in a 100 ml graduated cylinder fitted with a rubber stopper containing a sipper tube with a metal ball bearing to minimize spillage. Alcohol cylinders and water bottles were placed onto opposite sides of a standard cage lid and weighed before and after every 24 h session. The position of the water bottle and ethanol cylinder was switched at the beginning of every session to control side-preferences. A filled ethanol cylinder and water bottle were placed onto two empty cages and weighed to monitor spillage. The average spillage as a result of handling the cylinder and bottle was subtracted from the rats daily consumption. Rats were weighed every other day before receiving ethanol cylinders. Home-cage alcohol exposure was conducted identically for all experiments in this chapter except for experiment 7, as rats used for electrophysiological experiments were not exposed to alcohol.

Habituation

On the last day of home-cage alcohol exposure, rats were brought to the behavioural testing room in their home-cages and individually handled. On the two subsequent days all rats were habituated to Context 1 and then 24 h later to Context 2 in the conditioning chambers. Context 1 consisted of black walls, a clear Plexiglas floor, and a lemon odour. Context 2 consisted of clear Plexiglas walls, a wire-mesh floor, and an almond odour. Odours (3 sprays) were applied to a petri dish placed in the waste-pan under the chamber floor. Entries into a fluid port in the conditioning chamber were recorded during each context habituation session. All habituation sessions were 20 min long. Behaviour room and conditioning chamber habituation sessions occurred at the same time of day as subsequent training and test sessions.

Pavlovian conditioning with context alternation

Rats were assigned to context 1 or 2 for Pavlovian conditioning sessions ('alcohol context'), while the other context served as the 'neutral context'. Discrete stimuli were a 10 s, continuous white noise or 10 s clicker (5 Hz). One stimulus (conditioned stimulus, CS) was paired with alcohol delivery in the alcohol context and the other (neutral stimulus, NS) was presented without alcohol in the neutral context. Rats were counterbalanced across contexts, stimulus identity, and session order such that there were no differences in home-cage alcohol consumption. Rats then received one training session per day (73.5 min) that alternated between each context until 12 sessions of Pavlovian conditioning in the alcohol context and 12 sessions of exposure to the NS in the neutral context had occurred.

During training sessions, rats received 15 stimulus presentations (either CS or NS as per the appropriate context) with intervals of 140, 260, or 380 s between trials [mean variable time inter-trial interval (ITI) = 260 s]. In the alcohol context, CS presentations co-terminated with 6 s of syringe pump operation to deliver 0.2 ml of alcohol into the fluid port. In the neutral context, NS presentations also co-terminated with 6 s of syringe pump operation, but no alcohol was delivered.

The protocol for Pavlovian conditioning with context alternation was conducted identically in all experiments in this chapter, with 2 exceptions. First, experiment 1 used a subset of rats from experiment 1 in chapter 1 that had already undergone Pavlovian conditioning, here these rats underwent only 6 sessions of retraining (3 in each context) which were the same as

Pavlovian conditioning with context alternation. Second, experiment 7 used rats that had not undergone Pavlovian conditioning.

Experiment 1. Effect of dopamine receptor antagonists on CS responding

Three weeks after the termination of experiment 1 from chapter 1, a subset of rats (*n*=11; CS/NS group) received 3 Pavlovian conditioning sessions in the alcohol contexts alternated with 3 sessions of exposure to the NS in the neutral context. All rats received two 1 ml/kg subcutaneous (s.c.) saline habituation injections, 15 min before each of the last two training sessions, one in each context. Next, three CS responding tests were conducted in the neutral context. Tests started 15 min after systemic s.c. administration of vehicle (saline), the dopamine D2-like receptor antagonist eticlopride (10 μg/kg), or the D1-like receptor antagonist SCH23390 (10 μg/kg)[85,106]. Tests were separated by a single Pavlovian conditioning session in the alcohol context and followed a within-subjects design.

Experiment 2. Effect of chemogenetic inhibition of VTA dopamine neurons on CS responding

On the last two sessions of Pavlovian conditioning with context alternation, TH::Cre rats expressing the inhibitory designer receptor (hM4di) in the VTA (*n*=12) received an intraperitoneal (i.p.) 1 ml/kg saline injection 30 min before being placed into the conditioning chamber outfitted as the alcohol or neutral context. Next, 3 CS responding tests in the neutral context were conducted 30 min following systemic i.p. injections of vehicle (5% DMSO in saline), CNO at 10 mg/kg, or CNO at 20 mg/kg[131,134]. Tests were separated by four retraining sessions, alternating between the alcohol and neutral contexts (two sessions per context).

Experiment 3: Potential off-target effects of CNO and its parent compound clozapine

On the last two sessions of Pavlovian conditioning with context alternation, TH::Cre rats expressing a control fluorescent protein (mCherry) in the VTA (*n*=13) received an i.p. 1 ml/kg saline habituation injection 30 min before being placed into the conditioning chamber outfitted as the alcohol or neutral context. Next, three CS responding tests in the neutral context were conducted 30 min after systemic i.p. injections of vehicle (5% DMSO in saline), 10 mg/kg CNO (the lowest effective dose to reduce CS port entries in experiment 2), or 0.1 mg/kg clozapine (a dose that could be produced by the reverse metabolism of 10 mg/kg CNO[135]). One retraining session in the alcohol context and one in the neutral context separated each of the four tests.

Experiment 4: Chemogenetic inhibition of a dopaminergic VTA-to-NAc core circuit

On the last two sessions of Pavlovian conditioning with context alternation, TH::Cre rats expressing the inhibitory designer receptor (hM4Di) in the VTA (*n*=8) received a saline microinfusion (0.15 μl over 1 min) into the NAc core 5-15 min before being placed into the conditioning chamber outfitted as the alcohol or neutral context. At 24 h after the last training session, the first of four tests for CS responding was conducted. A similar number of rats were tested in the alcohol or neutral context after receiving a vehicle (saline) or CNO (3 mM)[146,147] microinfusion (0.3 μl over 1 min) 5-15 min before the test[131,146,148]. One retraining session in the alcohol context and one in the neutral context separated each of the four tests.

Experiment 5: Chemogenetic inhibition of a dopaminergic VTA-to-NAc shell circuit

On the last two sessions of Pavlovian conditioning with context alternation, TH::Cre rats expressing the inhibitory designer receptor (hM4Di) in the VTA (*n*=11) received a saline habituation microinfusion (0.15 μl over 1 min) into the NAc shell before a training session in the alcohol or neutral context. At 24 h after the last training session, the first of four tests for CS responding was conducted. A similar number of rats were tested in the alcohol or neutral context after receiving a vehicle (saline) or CNO (3 mM)[146,147] microinfusion (0.3 μl over 1 min) 5-15 min before the test session. One retraining session in the alcohol context and one in the neutral context separated each of the four tests.

Experiment 6: Selectivity of designer receptors for TH positive VTA neurons

After Pavlovian conditioning with context alternation, TH::Cre rats (*n*=4) were deeply anesthetized with euthanyl™ (sodium pentabarbitol, 240 mg/kg, i.p.) and perfused with phosphate buffered saline (250 ml, PBS, 0.02 M, pH 7.2) and 4% paraformaldehyde in 0.02 M PBS (150 ml, pH 7.2). Brains were immediately removed, cryoprotected in a 4% paraformaldehyde - 30% sucrose solution (50 ml, 2-3 days), and then stored at -80°C. Brains were sectioned (40 μm thick) using a cryostat (-20°C) and thaw-mounted onto slides that were stored at -20°C.

For immunohistochemistry, slides were removed from the -20°C freezer, covered and allowed to dry, in a fumehood overnight. The entire slide, excluding the label, was then outlined with an ImmEdge™ hydrophobic barrier pen (Vector labs, #H-4000) and then washed twice by pipetting 500 μl of 0.01 M PBS onto the slide for 1 min. After the second wash, 500 μl of 10%

normal donkey serum (NDS, Sigma-Aldrich, #D9663) in 0.01 M PBS plus 0.3% Triton X-100 (PBST) was applied to the slide for 30 min. After a 1 min wash in 0.01 M PBS, 500 μl of mouse anti-mCherry (1:1000, Abcam, #ab125096) and rabbit anti-TH (1:100, EMD Millipore, #ab152) in 10% NDS PBST was applied to the slides and left to incubate for 48 h at room temperature. Slides were then washed 3 times with 500 μl of 0.01 M PBS for 5 min. Then, 500 μl of donkey anti-mouse IgG (H+L) alexa 594 (1:200, Jackson ImmunoResearch labs, #715-585-150) and donkey anti-rabbit IgG (H+L) alexa 488 (1:200, Jackson ImmunoResearch labs, #711-545-152) in 0.01 M PBS was applied to the slides and left to incubate for 24 h at room temperature. Slides were left to dry covered at room temperature for 2 h and then cover-slipped with vectasheild™ (Vector labs, #H-1200) and imaged immediately or stored at 4°C until imaging.

To verify that hM4Di was selectively expressed in TH^+ cells, 6 coronal sections spanning 1.2 mm through the VTA from each of 4 rats were imaged using a Nikon laser scanning C2 system. Sections were surveyed at 4X magnification using a 594 nm excitation filter cube epifluorescence to identify the coronal section with the most expansive mCherry fluorescence. This coronal section was then imaged at 4X magnification with 405 nm, 488 nm, and 561 nm lasers to capture a 3 channel (DAPI, mCherry, TH) image of the entire section. Then, 2-4 images per hemisphere were taken at 40X magnification to again capture a 3-channel image (~300x300 μm) for colocalization analyses.

Experiment 7: Functional validation of circuit-specific chemogenetic inhibition

Male, TH::Cre rats (*n*=5) received stereotaxic surgery to deliver VTA microinfusions of 1 μl of AAV8-hSyn-DIO-hM4D(Gi)-mCherry (*n*=5). At 4-6 weeks later, rats were anaesthetized with isoflurane and decapitated. Brains were rapidly extracted and submerged in an ice-cold HEPES-based artificial cerebrospinal fluid (ASCF) solution containing (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 20 HEPES, 30 $NaHCO_3$, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 12 N-acetyl-L-cysteine (NAC), 10 $MgSO_4$, and 0.5 $CaCl_2$ (pH adjusted to ≈7.3-7.4 using 10 M NaOH) saturated with 95% O_2/5% CO_2. Coronal slices (300 μm) containing the NAc were obtained using a vibratome (Leica, VT1200) and transferred to a warm (34˚C), high-choline incubation solution containing (in mM): 92 choline chloride, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 12 NAC, 10 $MgSO_4$, and 0.5 $CaCl_2$, where they recovered for 12 min. Subsequently, slices were incubated at room temperature, in a normal ACSF solution containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 dextrose and were allowed to recover

for a minimum of 1 h prior to experiments. Once transferred into the recording chamber, slices were perfused with normal ACSF at 2 ml/min and were visualized using an upright fluorescence microscope with a 40X water-immersion objective, differential interference contrast optics (Olympus, BX51WI), and XM-10 monochrome camera for viewing (Olympus, CellSens v1.8). mCherry fluorescence in the NAc core was verified at 4X and 40X magnification prior to recordings.

Whole-cell patch-clamp pipettes made from boroscillate glass (1.0 mm OD, 3-5 MΩ) were filled with a recording solution containing (in mM): 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 2 ATP-tris, 0.4 GTP-tris (pH adjusted to 7.25 using KOH, 270-280 mOsm) and were lowered onto visually-identified neurons in the NAc core. Tight seals were obtained (1.3-6.6 GΩ), and cells were allowed to stabilize in whole-cell configuration for 10 min prior to recordings. Recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices), digitized (Digidata 1440A, Molecular Devices), and were stored using pClamp 10.3 software (Molecular Devices). Access resistance was 19.9 ± 2.2 MΩ, and series resistance was uncompensated. All cells recorded had a resting membrane potential below -65 mV. Cellular input resistance, membrane capacitance, and access resistance were monitored during each recording condition.

Cells were initially selected based on visual criteria; medium spiny neurons and GABAergic interneurons possess smaller soma in comparison to cholinergic interneurons (8-20 μm vs 20-50 μm[149–151]) and any cells with soma >30 μm were not recorded from. GABAergic interneurons and medium spiny projection neurons MSNs of the accumbens core were differentiated electrophysiologically by injecting 500 ms hyperpolarizing and depolarizing current steps between -100 and 100 pA in 10 pA intervals from the holding potential of -70 mV. Peak input resistance was measured at the largest voltage change in response to a -100 pA pulse, and steady state input resistance was assessed just prior to the end of the current step. Action potential properties were measured from the first action potential evoked in response to positive current injection.

Synaptic responses were evoked using a bipolar stimulating electrode made from two tungsten electrodes (≈1 MΩ, FHC Inc.) placed approximately 30 μm from the recording electrode. Evoked AMPA-receptor-mediated excitatory postsynaptic currents (EPCSs) were recorded at -70 mV, near the resting membrane potential of MSNs of the NAc core [152] using

constant current stimulation pulses. For each cell, at least 10 consecutive synaptic responses free from artifacts or action potentials were averaged for each phase of the recordings. Dopamine release from VTA terminals to the NAc core is under tonic inhibition from aspiny GABAergic interneurons[153], and dopamine can also modify inhibition in the accumbens[152,154]. Because GABA neurotransmission can alter the excitability of medium spiny neurons, picrotoxin (50 μM) was included in the ACSF to block $GABA_A$-mediated inhibition and better assess the effects of CNO on VTA inputs to medium spiny neurons. Recordings were obtained before and after 5 min application of 1 μM CNO, and were also obtained after 20 min washout of CNO in the continued presence of picrotoxin. The amplitudes of averaged synaptic currents were measured using Clampfit 8.2 software (Molecular Devices) and normalized to the amplitude of responses recorded prior to CNO application.

Histology and imaging

Rats from experiments 2-6 were deeply anesthetized with euthanyl™ (sodium pentabarbitol, 240 mg/kg, i. p.) and perfused with PBS (250 ml, 0.02 M, pH 7.2) and 4% paraformaldehyde in 0.02 M PBS (150 ml, pH 7.2). Brains were immediately removed, cryoprotected a 4% paraformaldehyde 30% sucrose solution (50 ml, ~2-3 days), and then stored at -80°C until they were sectioned (40 μm thick) using a cryostat at -20°C. Nissl staining was conducted to assess histological placements of injector tips in the NAc core and shell. Unamplified mCherry signal was used to verify successful designer receptor expression by letting slides dry for 24 h after removal from the -20°C freezer and then coverslipping them with vectasheild™. mCherry fluorescence was assessed using a Leica DM4000B epifluorescence microscope at 5X and 10X magnification.

Analyses and Statistics

Software

Statistical analyses were conducted with SigmaPlot™ v12 or SPSS™ v20 and graphs were made with SigmaPlot™ v12.

Dependent variables

During home-cage alcohol consumption grams of ethanol consumed per kilograms of body weight (g/kg) was measured. In Pavlovian conditioning and test sessions the following variables were measured, the number of: port entries per session (total port entries), port entries

during the 10 s CS (CS port entries), port entries made between CS offset and the next CS onset (140, 260, or 380 s; NonCS port entries), and port entries in the 10 s preceding the CS (PreCS port entries).

Analyses

Data from all experiments were analyzed using a repeated-measures ANOVA, and followed-up with post-hoc Bonferroni-corrected t-tests, or Newman-Keuls multiple comparisons. A Huynh-Feldt correction was applied when sphericity was violated in these analyses. All analyses used an alpha level of p=0.05.

Results

Experiment 1. Effect of dopamine receptor antagonists on CS responding

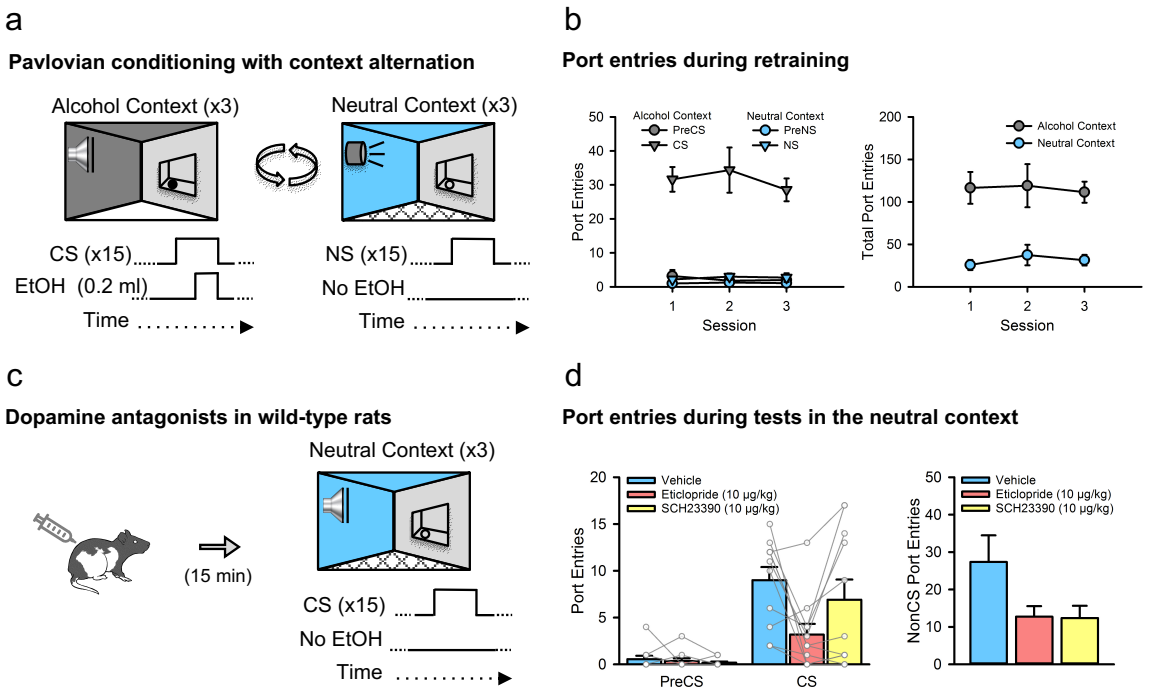
A subset of rats from experiment 1 in chapter 1 (n=11; CS/NS group) underwent retraining to re-establish the Pavlovian conditioning with context alternation they had previously experienced to pair a discrete CS with alcohol in a distinct alcohol context (**Fig 1a**). Responding during retraining was analyzed using a Session (1, 2, 3) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS, CS/NS) RM ANOVA. Port entries in the alcohol context were stably elevated compared to the neutral context [Session, $F_{(2, 20)}$=.951, p=.403; Context, $F_{(1, 10)}$=51.164, p<.001; Context x Session, $F_{(2, 20)}$=.594, p=.569]. The elevation in port entries in the alcohol context was driven by CS port entries as port entries during the PreCS remained stably low [Interval, $F_{(1, 10)}$=48.334, p<.001; Interval x Session, $F_{(2, 20)}$=1.357, p=.280]. Port entries during the NS, and PreNS intervals in the neutral context all remained stably low throughout retraining relative to CS port entries in the alcohol context which were stably elevated above PreCS port entries [Context x Interval, $F_{(1, 10)}$=46.269, p<.001; Interval x Context x Session, $F_{(2, 20)}$=1.337, p=.285; **Fig 1b** (left)].

Total port entries made during this retraining period were analyzed using a Session (1, 2, 3) by Context (Alcohol, Neutral) RM ANOVA. In the alcohol context, the total number of port entries were elevated relative to the neutral context [Context, $F_{(1, 10)}$=36.11, p<.001] and remained stable in both contexts [Session, $F_{(2, 20)}$=.174, p=.841; Session x Context, $F_{(2, 20)}$=.233, p=.794; **Fig 1b** (right)].

After an equal number of retraining sessions in each context, responding to the CS was tested in the absence of alcohol, in the neutral context on three subsequent days that were

intervened by retraining sessions in the alcohol context (**Fig 1c**). Test data were analyzed with an Interval (PreCS, CS) by Treatment (Vehicle, SCH23390, Eticlopride) within-subjects RM ANOVA. At test, rats made more port entries during the CS than the PreCS interval [Interval, $F_{(1, 10)}$=28.604, p<.001]. This elevated responding during the CS interval compared to the PreCS interval was reduced after drug pretreatment [Treatment, $F_{(2, 20)}$=5.162, p=.016; Interval x Treatment, $F_{(2, 20)}$=4.294, p=.028; **Fig 1d** (left)]. Follow-up Bonferroni-corrected t-tests were performed to assess which of the drug treatments differed from each other. Port entries made during the CS were significantly reduced from vehicle levels by pretreatment with Eticlopride [$t_{(10)}$= 3.56, p=.018; **Fig 1d** (left)] whereas SCH23390 did not reduce CS port entries compared to vehicle levels (p>1 after correction).

NonCS port entries at test were analyzed using a RM ANOVA including only the factor Treatment (Vehicle, SCH23390, Eticlopride). NonCS port entries made during the tests were significantly reduced following pretreatment with dopamine antagonists [Treatment, $F_{(2, 20)}$=4.17, p=.031; **Fig 1d** (right)].



Chapter 2 Figure 1: Experiment 1. Effect of dopamine receptor antagonists on CS responding. **a** First, rats (n=11; subset from chapter 1 figure 1) received 3 Pavlovian conditioning retraining sessions every other day in a distinct alcohol context wherein a discrete 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (3 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. **b** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral context across sessions of Pavlovian conditioning with context alternation (right). **c** After retraining, rats received the first of 3 tests for CS responding in the neutral context which were conducted on separate days and intervened by retraining sessions. Before tests, rats received a subcutaneous injection of vehicle, the D2-like receptor antagonist SCH23390 (10 µg/kg), or the D1-like receptor antagonist (10 µg/kg). **d** PreCS and CS port entries (left) and NonCS port entries (right) during tests in the neutral context. Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 1

Experiment 2. Effect of chemogenetic inhibition of VTA dopamine neurons on CS responding

To evaluate the impact of reducing activity in a subset of ventral tegmental area (VTA) dopamine neurons on responding to a discrete cue for alcohol a chemogenetic approach in transgenic rats was used. Naïve TH::Cre male rats (n=12) were microinfused bilaterally into the VTA with the double-floxed[132] inhibitory designer receptor construct[130] AAV8-hSyn-DIO-hM4Di-mCherry, resulting in the selective expression of the inhibitory designer receptor (hM4Di) in VTA dopamine neurons. This receptor inhibits neuronal firing when bound by the exogenous ligand clozapine-*n*-oxide (CNO)[130,131].

After recovering from surgery, rats received twelve sessions of exposure to alcohol in the home-cage (**Fig 2a**). The dependent measure for home-cage alcohol exposure was grams of ethanol consumed as a function of rat weight in kilograms (g/kg). Alcohol consumption was analyzed using a within-subjects RM ANOVA including the factor Session (1, 2, 3…12). Alcohol consumption increased across home-cage sessions [Session, $F_{(11, 132)}$=3.799, p<.001; **Fig 2b**].

Then, rats underwent Pavlovian conditioning with context alternation (**Fig 2c**). Port entries during Pavlovian conditioning with context alternation were analyzed in a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS, CS/NS) RM ANOVA. Port entries increased in the alcohol context relative to the neutral context across sessions [Session, $F_{(11, 121)}$=11.191, p<.001; Context, $F_{(1, 11)}$=38.223, p<.001; Context x Session, $F_{(11, 121)}$=12.935, p<.001]. The elevation in port entries in the alcohol context was driven by CS port entries which increased into a plateau, whereas port entries during the PreCS in the alcohol context remained stably low [Interval, $F_{(1, 11)}$=38.371, p<.001; Interval x Session, $F_{(11, 121)}$=13.464, p<.001]. Port entries during the NS and PreNS intervals in the neutral context remained stably low throughout conditioning relative to CS port entries in the alcohol context [Context x Interval, $F_{(1, 11)}$=28.738, p<.001]. Importantly, the increase in CS relative to PreCS port entries in the alcohol context, while port entries during all intervals in the neutral context remained low and stable, suggests that rats specifically acquired responding to the CS in the alcohol context [Interval x Context x Session, $F_{(11, 121)}$=8.876, p<.001; **Fig 2d** (left)].

The total number of port entries made during each training session was analyzed in a RM ANOVA including the factors Session (1, 2, 3, …12) and Context (Alcohol, Neutral). In the alcohol context, the total number of port entries were elevated relative to the neutral context

[Context, $F_{(1, 11)}$=129.409, p<.001], wherein port entries declined across sessions [Session, $F_{(11, 121)}$=3.029, p=.001; Context x Session, $F_{(11, 121)}$=3.054, p=.001; **Fig 2d** (right)].

Next, CS responding was tested by presenting the CS without alcohol in the neutral context 30 min after a systemic i.p. injection of vehicle or CNO (10 mg/kg or 20 mg/kg; **Fig 2e**)[131,155]. Test data were analyzed with an Interval (PreCS, CS) by Treatment (Vehicle, CNO 10 mg/kg, 20 mg/kg) within-subjects RM ANOVA and NonCS port entries were analyzed using a separate RM ANOVA including only the factor Treatment (Vehicle, CNO 10 mg/kg, 20 mg/kg). PreCS port entries were minimal at test compared to CS port entries [Interval, $F_{(1, 11)}$=62.444, p<.001] which were reduced by CNO [Treatment, $F_{(2, 22)}$=10.592, p=.001; Interval x Treatment, $F_{(2, 22)}$=10.842, p=.001; **Fig 2f** (left)]. Post-hoc Bonferroni-corrected t-tests revealed that CS port entries were significantly reduced following systemic administration of 10 mg/kg and 20 mg/kg of CNO [$t_{(11)}$=3.171, p=.03 for 10 mg/kg, and $t_{(11)}$=3.644, p=.012 for 20 mg/kg, both relative to vehicle]. However, CS responding after treatment with the CNO doses did not differ from one another (p>1 after correction). The reduction in CS port entries could not be explained by general decreases in port directed behaviour, as NonCS port entries were similar across all tests [Treatment, $F_{(2, 22)}$=.288, p=.752; **Fig 2f** (right)].

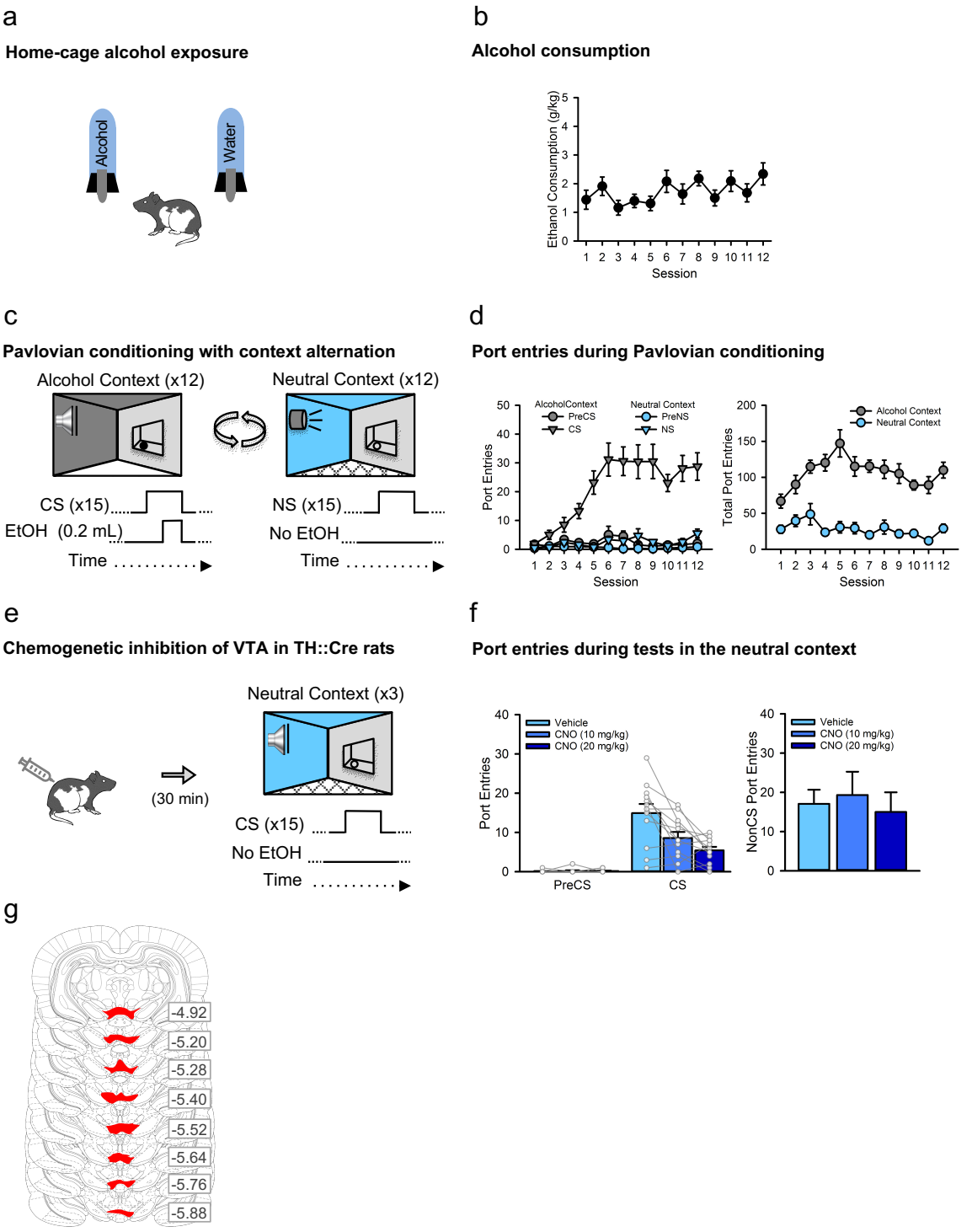

a
Home-cage alcohol exposure
Alcohol
Water
b
Alcohol consumption
Ethanol Consumption (g/kg)
Session
c
Pavlovian conditioning with context alternation
Alcohol Context (x12)
Neutral Context (x12)
CS (x15)
EtOH (0.2 mL)
Time
NS (x15)
No EtOH
Time
d
Port entries during Pavlovian conditioning
AlcoholContext
PreCS
CS
Neutral Context
PreNS
NS
Port Entries
Session
Alcohol Context
Neutral Context
Total Port Entries
Session
e
Chemogenetic inhibition of VTA in TH::Cre rats
(30 min)
Neutral Context (x3)
CS (x15)
No EtOH
Time
f
Port entries during tests in the neutral context
Vehicle
CNO (10 mg/kg)
CNO (20 mg/kg)
Port Entries
PreCS
CS
Vehicle
CNO (10 mg/kg)
CNO (20 mg/kg)
NonCS Port Entries
g
-4.92
-5.20
-5.28
-5.40
-5.52
-5.64
-5.76
-5.88

Figure 2

Chapter 2 Figure 2: Experiment 2. Effect of chemogenetic inhibition of VTA dopamine neurons on CS responding. **a** After recovering from surgery wherein TH::Cre rats (n=12) received microinfusion of a viral construct encoding for the inhibitory designer receptor (AAV8-hSyn-DIO-hM4Di-mCherry) in the ventral tegmental area (VTA), rats received 24 h access to a 15% ethanol (alcohol) solution in their home-cage every other day for a total of 12 sessions. Water and standard rat chow were continuously available. **b** Alcohol consumption shown as grams of ethanol over kilograms of body weight across 12 home-cage alcohol exposure sessions. **c** Then, rats received 12 Pavlovian conditioning sessions every other day in a distinct alcohol context wherein a discrete, 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (12 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. **d** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral context across sessions of Pavlovian conditioning with context alternation (right). **e** After Pavlovian conditioning with context alternation, rats received the first of 3 tests for CS responding in the neutral context which were conducted on separate days and intervened by retraining sessions. Before tests, rats received an intraperitoneal injection of vehicle or CNO (10 mg/kg or 20 mg/kg). **f** PreCS and CS port entries (left) and NonCS port entries (right) during tests in the neutral context. **g** Maximal mCherry expression in the VTA for each rat is shown in schematics from the atlas of Paxinos & Watson (2008). Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 2

Experiment 3: Potential off-target effects of CNO and its parent compound clozapine

To investigate the potential off-target effects of CNO or its parent compound clozapine on behaviour, a naïve group of 13 TH::Cre rsts were microinfused bilaterally into the VTA with the double-floxed[132] control construct AAV8-hSyn-DIO-mCherry, resulting in the selective expression of the mCherry protein in VTA dopamine neurons. These rats were then tested for CS responding in the neutral context after receiving the lowest effective dose of CNO (10 mg/kg) from experiment 2, or clozapine at a dose (0.1 mg/kg) that may result from the reverse metabolism of 10 mg/kg CNO[135], according to a within-subject design.

After recovering from surgery, rats received twelve sessions of exposure to alcohol in the home-cage (**Fig 3a**). The dependent measure for home-cage alcohol exposure was grams of ethanol consumed as a function of rat weight in kilograms (g/kg). Alcohol consumption was analyzed using a within-subjects RM ANOVA including the factor Session (1, 2, 3…12). Alcohol consumption increased across home-cage sessions [Session, $F_{(11, 154)}$=4.139, p<.001; **Fig 3b**].

Then, rats underwent Pavlovian conditioning with context alternation (**Fig 3c**). Port entries during Pavlovian conditioning with context alternation were analyzed using a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS or CS/NS) RM ANOVA. Port entries in the alcohol context increased across sessions and were elevated relative to the neutral context [Session, $F_{(11, 132)}$=9.403, p<.001; Context, $F_{(1, 12)}$=46.116, p<.001; Context x Session, $F_{(11, 132)}$=11.613, p<.001]. The elevation in port entries in the alcohol context was driven by CS port entries which increased into a plateau, whereas port entries during the PreCS remained stably low [Interval, $F_{(1, 12)}$=45.953, p<.001; Interval x Session, $F_{(11, 132)}$=14.049, p<.001]. Port entries during the NS and PreNS intervals in the neutral context all remained stably low throughout conditioning relative to CS port entries in the alcohol context [Context x Interval, $F_{(1, 12)}$=39.140, p<.001]. Importantly, the increase in CS relative to PreCS port entries in the alcohol context, while port entries during all intervals in the neutral context remained low and stable, suggests that rats specifically acquired responding to the CS in the alcohol context [Interval x Context x Session, $F_{(11, 132)}$=11.323, p<.001; **Fig 3d** (left)].

The total number of port entries made during each training session was analyzed using a RM ANOVA including the factors Session (1, 2, 3, …12) and Context (Alcohol, Neutral). In the alcohol context, the total number of port entries were elevated relative to the neutral context [Context, $F_{(1, 12)}$=78.793, p<.001] and total port entries in both contexts were generally stable

across sessions [Session, $F_{(11, 121)}$=1.151, p=.328; Context x Session, $F_{(11, 132)}$=1.034, p=.421; **Fig 3d** (right)].

Next, CS responding was tested by presenting the CS without alcohol in the neutral context 30 min after a systemic i.p. injection of vehicle, CNO (10 mg/kg), or Clozapine (0.1 mg/kg; **Fig 3e**)[131,155]. Test data were analyzed with an Interval (PreCS, CS) by Treatment (Vehicle, CNO 10 mg/kg, Clozapine 0.1 mg/kg) RM ANOVA and NonCS port entries were analyzed in a separate RM ANOVA including only the factor Treatment (Vehicle, CNO 10 mg/kg, Clozapine 0.1 mg/kg). PreCS port entries were minimal at test compared to CS port entries [Interval, $F_{(1, 12)}$=57.777, p<.001] and responding during both intervals was unaffected by CNO or clozapine [Treatment, $F_{(2, 22)}$=.339, p=.716; Interval x Treatment, $F_{(2, 24)}$=.431, p=.655; **Fig 3f** (left)]. Port entries made between CS trials, NonCS port entries, were similarly unaffected by CNO or clozapine [Treatment, $F_{(2, 24)}$=1.678, p=.208; **Fig 3f** (right)]. Thus, the suppression of CS responding produced by chemogenetically inhibiting VTA dopamine neurons (Exp. 2) could not be accounted for by non-specific effects of systemically injected CNO or its reverse-metabolism to clozapine. Together, these results support the hypothesis that VTA dopamine neuron activity is necessary for responding to a discrete alcohol cue in a neutral context.

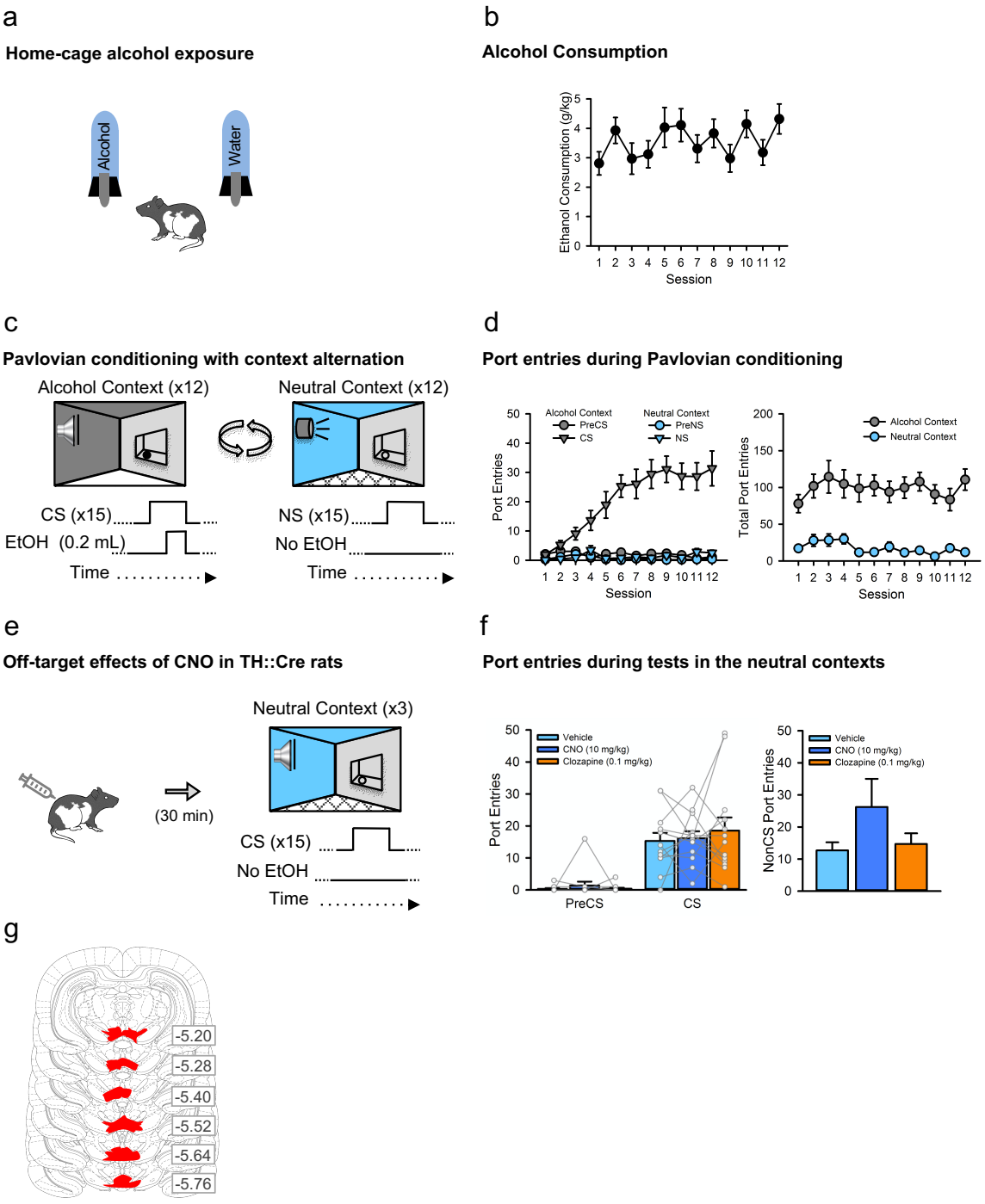

a
Home-cage alcohol exposure
Alcohol
Water
b
Alcohol Consumption
Ethanol Consumption (g/kg)
Session
c
Pavlovian conditioning with context alternation
Alcohol Context (x12)
Neutral Context (x12)
CS (x15)
EtOH (0.2 mL)
Time
NS (x15)
No EtOH
Time
d
Port entries during Pavlovian conditioning
Alcohol Context
PreCS
CS
Neutral Context
PreNS
NS
Port Entries
Session
Alcohol Context
Neutral Context
Total Port Entries
Session
e
Off-target effects of CNO in TH::Cre rats
Neutral Context (x3)
(30 min)
CS (x15)
No EtOH
Time
f
Port entries during tests in the neutral contexts
Vehicle
CNO (10 mg/kg)
Clozapine (0.1 mg/kg)
Port Entries
PreCS
CS
Vehicle
CNO (10 mg/kg)
Clozapine (0.1 mg/kg)
NonCS Port Entries
g
-5.20
-5.28
-5.40
-5.52
-5.64
-5.76

Figure 3

Chapter 2 Figure 3: Experiment 3: Potential off-target effects of CNO and its parent compound clozapine. **a** After recovering from surgery wherein TH::Cre rats (n=13) received microinfusion of a viral construct encoding for a control fluorescent protein (AAV8-hSyn-DIO-mCherry) in the ventral tegmental area (VTA), rats received 24 h access to a 15% ethanol (alcohol) solution in their home-cage every other day for a total of 12 sessions. Water and standard rat chow were continuously available. **b** Alcohol consumption shown as grams of ethanol over kilograms of body weight across 12 home-cage alcohol exposure sessions. **c** Then, rats received 12 Pavlovian conditioning sessions every other day in a distinct alcohol context wherein a discrete, 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (12 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. **d** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral context across sessions of Pavlovian conditioning with context alternation (right). **e** After Pavlovian conditioning with context alternation, rats received the first of 3 tests for CS responding in the neutral context which were conducted on separate days and intervened by retraining sessions. Before tests, rats received an intraperitoneal injection of vehicle, CNO (10 mg/kg), or clozapine (0.1 mg/kg). **f** PreCS and CS port entries (left) and NonCS port entries (right) during tests in the neutral context. **g** Maximal mCherry expression in the VTA for each rat is shown in schematics from the atlas of Paxinos & Watson (2008). Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 3

Experiment 4: Chemogenetic inhibition of a dopaminergic VTA-to-NAc core circuit

A circuit-specific chemogenetic approach was used to test the prediction that a dopaminergic projection from the VTA to the NAc core was necessary for responding to a discrete alcohol cue, irrespective of context. The dopaminergic VTA-to-NAc core projection was targeted by microinfusing the viral AAV8-hSyn-DIO-hM4Di-mCherry construct into the VTA of naïve TH::Cre rats and implanting bilateral cannulae above the NAc core. The dopaminergic VTA-to-NAc core projection was then inhibited by microinfusing CNO (0.3 µl, 3 mM) through injectors targeting the NAc core.

After recovering from surgery, rats received twelve sessions of exposure to alcohol in the home-cage (**Fig 4a**). The dependent measure for home-cage alcohol exposure was grams of ethanol consumed as a function of rat weight in kilograms (g/kg). Alcohol consumption was analyzed using a within-subjects RM ANOVA including the factor Session (1, 2, 3…12). Alcohol consumption increased across home-cage sessions [Session, $F_{(11, 77)}$=2.257, p=.019; **Fig 4b**].

Then, rats underwent Pavlovian conditioning with context alternation (**Fig 4c**). Port entries during Pavlovian conditioning with context alternation were analyzed using a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS or CS/NS) RM ANOVA. Port entries increased and were elevated in the alcohol context relative to the neutral context [Session, $F_{(11, 77)}$=6.165, p<.001; Context, $F_{(1, 7)}$=17.880, p=.004; Context x Session, $F_{(11, 77)}$=5.701, p<.001]. The elevation in port entries in the alcohol context was driven by CS port entries which increased into a plateau, whereas port entries during the PreCS remained stably low [Interval, $F_{(1, 7)}$=15.468, p=.006; Interval x Session, $F_{(11, 77)}$=7.103, p=.001]. Port entries during the NS, and PreNS intervals in the neutral context all remained stably low throughout conditioning relative to CS port entries in the alcohol context [Context x Interval, $F_{(1, 7)}$=13.973, p=.007]. Importantly, the increase in CS relative to PreCS port entries in the alcohol context, while port entries during all intervals in the neutral context remained low and stable, suggests that rats specifically acquired responding to the CS in the alcohol context [Interval x Context x Session, $F_{(11, 77)}$=5.352, p<.001; **Fig 4d** (left)].

The total number of port entries made during each training session was analyzed in a RM ANOVA including the factors Session (1, 2, 3, …12) and Context (Alcohol, Neutral). In the alcohol context, the total number of port entries were elevated relative to the neutral context [Context, $F_{(1, 17)}$=47.187, p<.001]. Responding varied from session to session at the beginning of

training until behaviour stabilized [Session, $F_{(11, 77)}$=2.455, p=.011; Context x Session, $F_{(11, 77)}$=1.605, p=.114; **Fig 4d** (right)].

After training, CS responding was tested by presenting the CS without alcohol in the alcohol and neutral context, after a microinfusion of vehicle or CNO according to a within-subjects design (**Fig 4e**). Test data were analyzed with an Interval (PreCS, CS) by Treatment (Vehicle, CNO) by Context (Alcohol, Neutral) RM ANOVA and NonCS port entries were analyzed in a separate RM ANOVA including the factors Treatment (Vehicle, CNO) and Context (Alcohol, Neutral). At test, CS port entries were elevated over PreCS port entries [Interval, $F_{(1, 7)}$=553.730, p<.001], a difference which was larger in the alcohol than the neutral context [Context, $F_{(1, 7)}$=40.560, p<.001, Context x Interval, $F_{(1, 7)}$=44.840, p<.001; **Fig 4f** (left)]. CNO microinfusion into the NAc core reduced CS port entries in both the alcohol and neutral contexts [Treatment, $F_{(1, 7)}$=6.324, p=.04; Treatment x Interval, $F_{(1, 7)}$=5.763, p=.047; Context x Treatment, $F_{(1, 7)}$=.006, p=.940; Context x Treatment x Interval, $F_{(1, 7)}$=.002, p=.962; **Fig 4f** (left)]. NonCS port entries were unaffected by CNO or context [Treatment, $F_{(1, 7)}$=.063, p=.809; Context x Treatment, $F_{(1, 7)}$=.688, p=.434; Context, $F_{(1, 7)}$=4.307, p=.077; **Fig 4f** (right)].

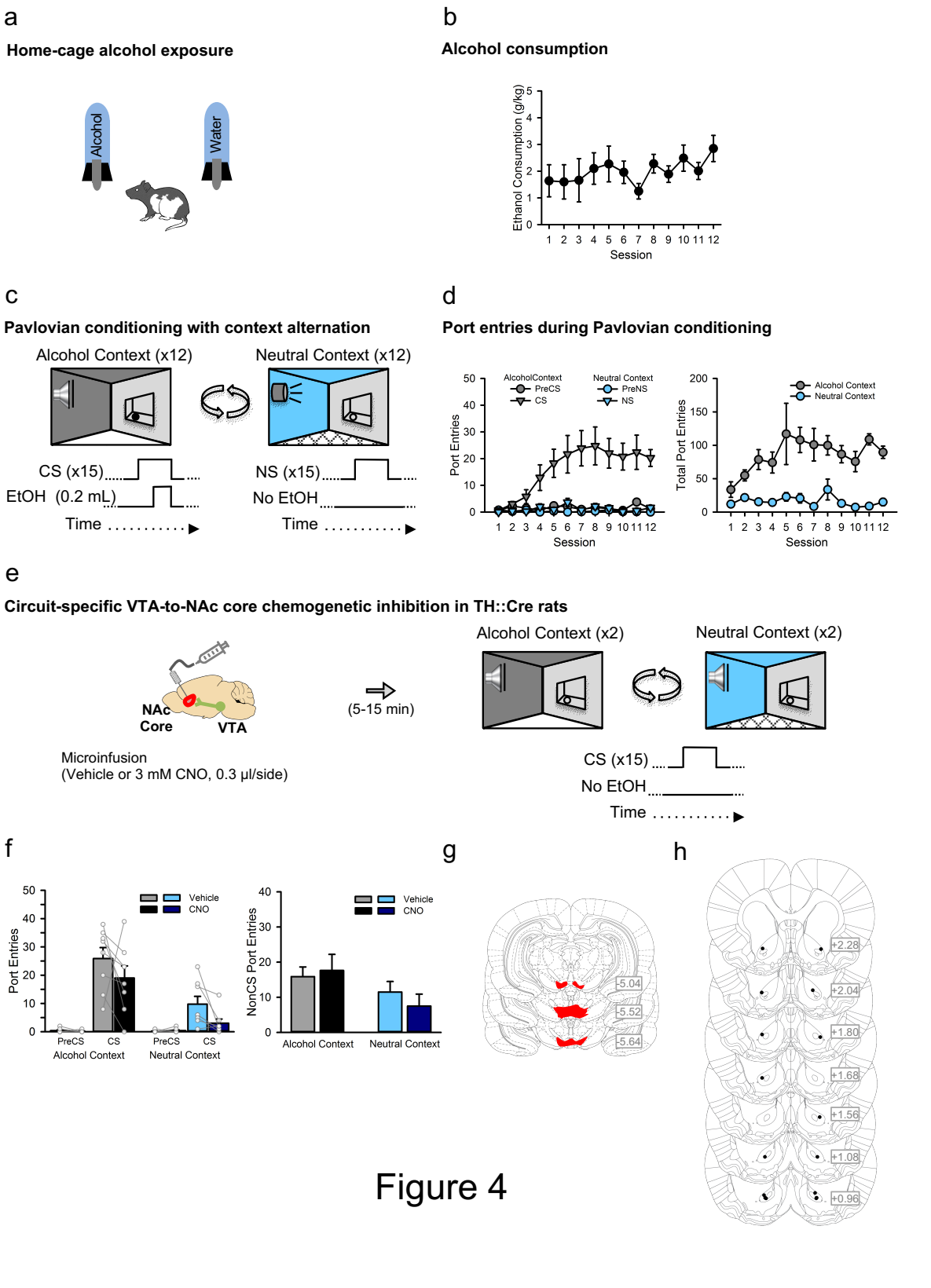

a
Home-cage alcohol exposure
Alcohol
Water
b
Alcohol consumption
Ethanol Consumption (g/kg)
Session
c
Pavlovian conditioning with context alternation
Alcohol Context (x12)
Neutral Context (x12)
CS (x15)
EtOH (0.2 mL)
Time
NS (x15)
No EtOH
Time
d
Port entries during Pavlovian conditioning
AlcoholContext
PreCS
CS
Neutral Context
PreNS
NS
Port Entries
Session
Alcohol Context
Neutral Context
Total Port Entries
Session
e
Circuit-specific VTA-to-NAc core chemogenetic inhibition in TH::Cre rats
NAc Core
VTA
Microinfusion
(Vehicle or 3 mM CNO, 0.3 µl/side)
(5-15 min)
Alcohol Context (x2)
Neutral Context (x2)
CS (x15)
No EtOH
Time
f
Vehicle
CNO
Port Entries
PreCS
CS
PreCS
CS
Alcohol Context
Neutral Context
NonCS Port Entries
Alcohol Context
Neutral Context
g
-5.04
-5.52
-5.64
h
+2.28
+2.04
+1.80
+1.68
+1.56
+1.08
+0.96

Figure 4

Chapter 2 Figure 4: Experiment 4: Chemogenetic inhibition of a dopaminergic VTA-to-NAc core circuit. **a** After recovering from surgery wherein TH::Cre rats (n=8) received microinfusion of a viral construct encoding the inhibitory designer receptor (AAV8-hSyn-DIO-hM4Di-mCherry) in the ventral tegmental area (VTA) and cannulae targeting the nucleus accumbens (NAc) core, rats received 24 h access to a 15% ethanol (alcohol) solution in their home-cage every other day for a total of 12 sessions. Water and standard rat chow were continuously available. **b** Alcohol consumption shown as grams of ethanol over kilograms of body weight across 12 home-cage alcohol exposure sessions. **c** Then, rats received 12 Pavlovian conditioning sessions every other day in a distinct alcohol context wherein a discrete, 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (12 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. **d** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral context across sessions of Pavlovian conditioning with context alternation (right). **e** After Pavlovian conditioning with context alternation, rats received the first of 4 tests (2 in each context) for CS responding in the alcohol and neutral contexts which were conducted on separate days and intervened by retraining sessions. Before tests, rats received intracranial microinfusion of vehicle or CNO (0.3 µl, 3 mM, 0.3 µl/min). **f** PreCS and CS port entries (left) and NonCS port entries (right) during tests in the alcohol and neutral context. **g** Maximal mCherry expression in the VTA and **h** injector placements in the NAc core for each rat is shown in schematics from the atlas of Paxinos & Watson (2008). Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 4

Experiment 5: Chemogenetic inhibition of a dopaminergic VTA-to-NAc shell circuit

A circuit-specific chemogenetic approach was used to test the prediction that a dopaminergic projection from the VTA to the NAc shell was necessary for the elevation of CS responding in the alcohol context, but not for CS responding in the neutral context. The dopaminergic VTA-to-NAc shell projection was targeted by microinfusing the viral AAV8-hSyn-DIO-hM4Di-mCherry construct into the VTA of naïve TH::Cre rats and implanting bilateral cannulae above the NAc shell. The dopaminergic VTA-to-NAc shell projection was then inhibited by microinfusing CNO (3 µl, 3 mM) through injectors targeting the NAc shell.

After recovering from surgery, rats received twelve sessions of exposure to alcohol in the home-cage (**Fig 5a**). The dependent measure for home-cage alcohol exposure was grams of ethanol consumed as a function of rat weight in kilograms (g/kg). Alcohol consumption was analyzed using a RM ANOVA including the factor Session (1, 2, 3…12). Alcohol consumption increased across home-cage sessions [$F_{(11, 110)}$=7.140, p<.001; **Fig 5b**].

Then, rats underwent Pavlovian conditioning with context alternation (**Fig 5c**). Port entries during Pavlovian conditioning with context alternation were analyzed using a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS or CS/NS) RM ANOVA. Port entries increased in the alcohol context relative to the neutral context [Session, $F_{(11, 110)}$=7.018, p<.001; Context, $F_{(1, 10)}$=13.001, p=.005; Context x Session, $F_{(11, 110)}$=7.252, p<.001]. The elevation in port entries in the alcohol context was driven by CS port entries which increased into a plateau, whereas port entries during the PreCS remained stably low [Interval, $F_{(1, 10)}$=12.197, p=.006; Interval x Session, $F_{(11, 110)}$=8.273, p<.001]. Port entries during the NS, and PreNS intervals in the neutral context all remained stably low throughout conditioning relative to CS port entries in the alcohol context [Context x Interval, $F_{(1, 10)}$=11.785, p=.006]. Importantly, the increase in CS relative to PreCS port entries in the alcohol context, while port entries during all intervals in the neutral context remained low and stable, suggests that rats specifically acquired responding to the CS in the alcohol context [Interval x Context x Session, $F_{(11, 110)}$=8.149, p<.001; **Fig 5d** (left)].

The total number of port entries made during each training session was analyzed in a RM ANOVA including the factors Session (1, 2, 3, …12) and Context (Alcohol, Neutral). In the alcohol context, the total number of port entries were elevated relative to the neutral context [Context, $F_{(1, 10)}$=25.726, p<.001]. Total port entries decreased to low levels across training

sessions in the neutral context relative to the alcohol context [Session, $F_{(11, 110)}$=1.355, p=.205; Context x Session, $F_{(11, 110)}$=2.235, p=.017; **Fig 5d** (right)].

After training, CS responding was tested by presenting the CS without alcohol in the alcohol and neutral context, after a microinfusion of vehicle or CNO according to a within-subjects design (**Fig 5e**). Test data were analyzed with an Interval (PreCS, CS) by Treatment (Vehicle, CNO) by Context (Alcohol, Neutral) RM ANOVA and NonCS port entries were analyzed in a separate RM ANOVA including the factors Treatment (Vehicle, CNO) and Context (Alcohol, Neutral). At test, CS port entries were elevated over PreCS port entries [Interval, $F_{(1, 10)}$=28.226, p<.001] a difference which was larger in the alcohol than the neutral context [Context, $F_{(1, 10)}$=17.524, p<.001; Context x Interval, $F_{(1, 10)}$=9.562, p=.011; **Fig 5f** (left)]. CNO microinfusion into the NAc shell reduced CS port entries in the alcohol context but not the neutral context [Treatment, $F_{(1, 10)}$=3.418, p=.094; Treatment x Interval, $F_{(1, 10)}$=6.244, p=.032; Context x Treatment, $F_{(10)}$=5.887, p=.036; Context x Treatment x Interval, $F_{(1, 10)}$=5.121, p=.047; **Fig 5f** (left)]. Post-hoc Bonferroni-corrected t-tests confirmed that CS port entries were significantly lower in the alcohol context after CNO microinfusion [$t_{(10)}$=3.121, p=.011] but not in the neutral context [$t_{(10)}$=-.367, p=.721]. NonCS port entries were significantly elevated in the alcohol context relative to the neutral context [Context, $F_{(1, 10)}$=16.221, p=.002] but were unaffected by CNO [Treatment, $F_{(1, 10)}$=.03, p=.866] in either context [Treatment x Context, $F_{(1, 10)}$=1.093, p=.32; **Fig 5f** (right)].

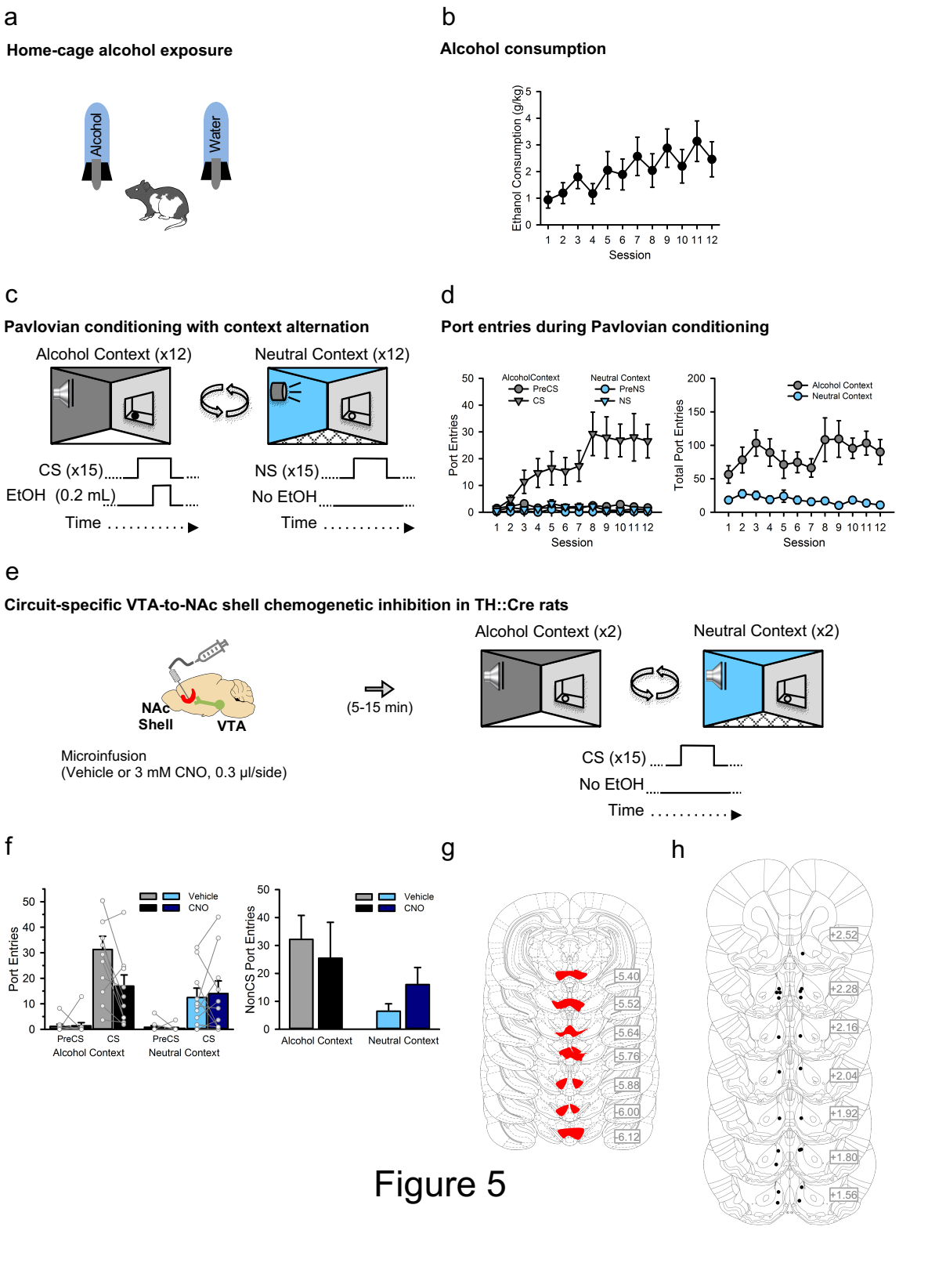

a
Home-cage alcohol exposure
Alcohol
Water
b
Alcohol consumption
Ethanol Consumption (g/kg)
Session
c
Pavlovian conditioning with context alternation
Alcohol Context (x12)
Neutral Context (x12)
CS (x15)
EtOH (0.2 mL)
Time
NS (x15)
No EtOH
Time
d
Port entries during Pavlovian conditioning
AlcoholContext
PreCS
CS
Neutral Context
PreNS
NS
Port Entries
Session
Alcohol Context
Neutral Context
Total Port Entries
Session
e
Circuit-specific VTA-to-NAc shell chemogenetic inhibition in TH::Cre rats
NAc Shell
VTA
Microinfusion
(Vehicle or 3 mM CNO, 0.3 µl/side)
(5-15 min)
Alcohol Context (x2)
Neutral Context (x2)
CS (x15)
No EtOH
Time
f
Vehicle
CNO
Port Entries
PreCS
CS
PreCS
CS
Alcohol Context
Neutral Context
Vehicle
CNO
NonCS Port Entries
Alcohol Context
Neutral Context
g
-5.40
-5.52
-5.64
-5.76
-5.88
-6.00
-6.12
h
+2.52
+2.28
+2.16
+2.04
+1.92
+1.80
+1.56

Figure 5

Chapter 2 Figure 5: Experiment 5: Chemogenetic inhibition of a dopaminergic VTA-to-NAc shell circuit. **a** After recovering from surgery wherein TH::Cre rats (n=11) received microinfusion of a viral construct encoding the inhibitory designer receptor (AAV8-hSyn-DIO-hM4Di-mCherry) in the ventral tegmental area (VTA) and cannulae targeting the nucleus accumbens (NAc) shell, rats received 24 h access to a 15% ethanol (alcohol) solution in their home-cage every other day for a total of 12 sessions. Water and standard rat chow were continuously available. **b** Alcohol consumption shown as grams of ethanol over kilograms of body weight across 12 home-cage alcohol exposure sessions. **c** Then, rats received 12 Pavlovian conditioning sessions every other day in a distinct alcohol context wherein a discrete, 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (12 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. **d** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral context across sessions of Pavlovian conditioning with context alternation (right). **e** After Pavlovian conditioning with context alternation, rats received the first of 4 tests (2 in each context) for CS responding in the alcohol and neutral contexts which were conducted on separate days and intervened by retraining sessions. Before tests, rats received intracranial microinfusion of vehicle or CNO (0.3 µl, 3 mM, 0.3 µl/min). **f** PreCS and CS port entries (left) and NonCS port entries (right) during tests in the alcohol and neutral context. **g** Maximal mCherry expression in the VTA and **h** injector placements in the NAc shell for each rat is shown in schematics from the atlas of Paxinos & Watson (2008). Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 5

Experiment 6: Selectivity of designer receptors for TH positive VTA neurons

To validate the selectivity of Cre for TH positive neurons, and the transfection efficiency of the AAV8-hSyn-DIO-hM4Di-mCherry construct in TH::Cre rats[130,132,133] brains from 4 TH::Cre$^{+/-}$ rats that received home-cage alcohol exposure and Pavlovian conditioning with context alternation were used for immunohistochemical labeling of TH and amplification of the mCherry signal. In a single optical plane through the VTA at approximately bregma -5.5 mm (**Fig 6a, d**), 12.0 ± 1.5 cells were mCherry positive, 52.3 ± 6.3 were TH positive, and 11.6 ± 1.5 were colocalized (**Fig 6b**). These counts produced an average selectivity of mCherry-expression for TH positive cells of 95.8% and a transfection efficiency of 24.8% (**Fig 6c**), which are comparable to similar measures of selectivity in TH::Cre rats[67,131,133,143,156,157]. Importantly, mCherry expression was also observed in TH positive terminals near injector tips in the NAc core (Exp. 4; **Fig 6e-h**) and shell (Exp. 5; **Fig 6i-l**).

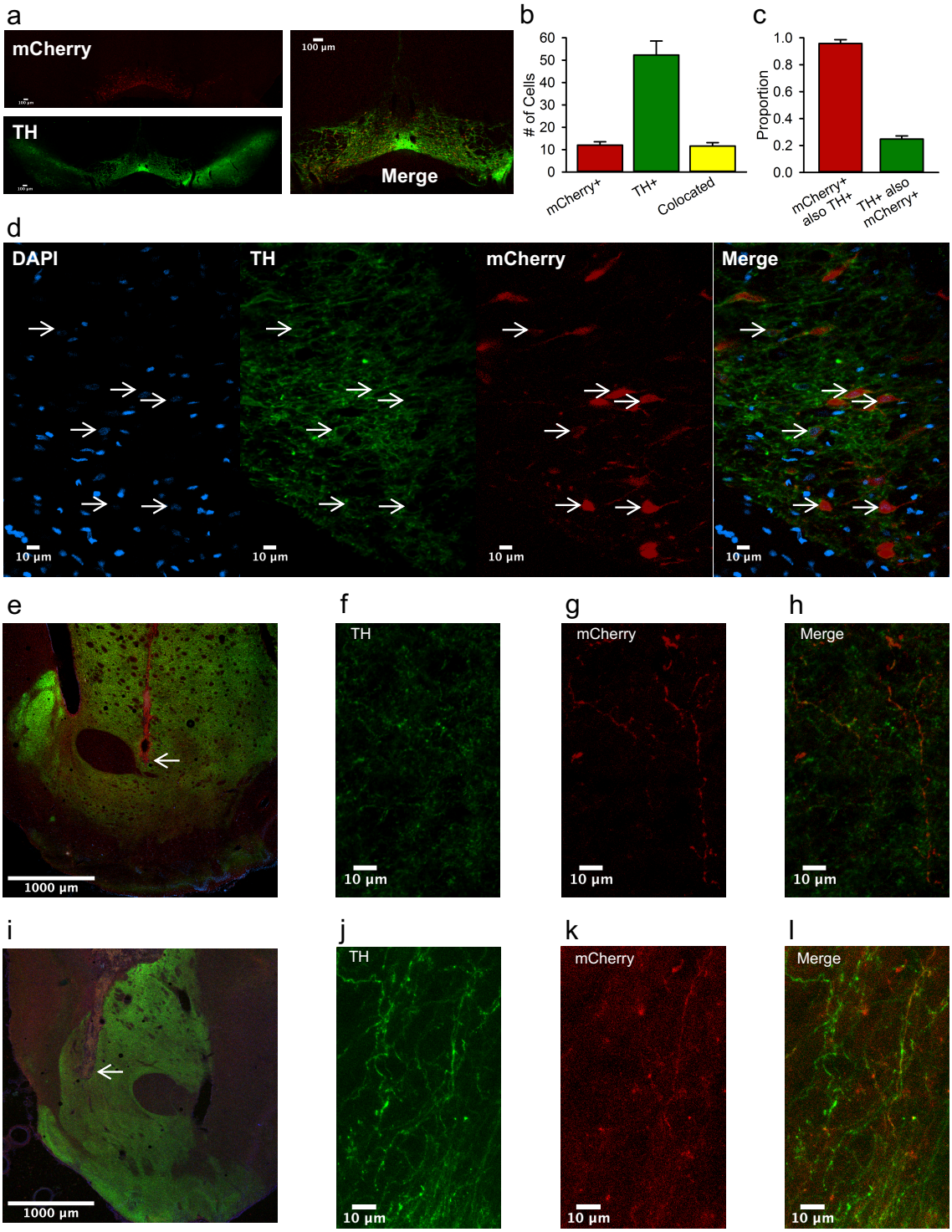

a
mCherry
TH
100 µm
Merge
b
# of Cells
60
50
40
30
20
10
0
mCherry+
TH+
Colocated
c
Proportion
1.0
0.8
0.6
0.4
0.2
0.0
mCherry+ also TH+
TH+ also mCherry+
d
DAPI
TH
mCherry
Merge
10 µm
e
1000 µm
f
TH
10 µm
g
mCherry
10 µm
h
Merge
10 µm
i
1000 µm
j
TH
10 µm
k
mCherry
10 µm
l
Merge
10 µm

Figure 6

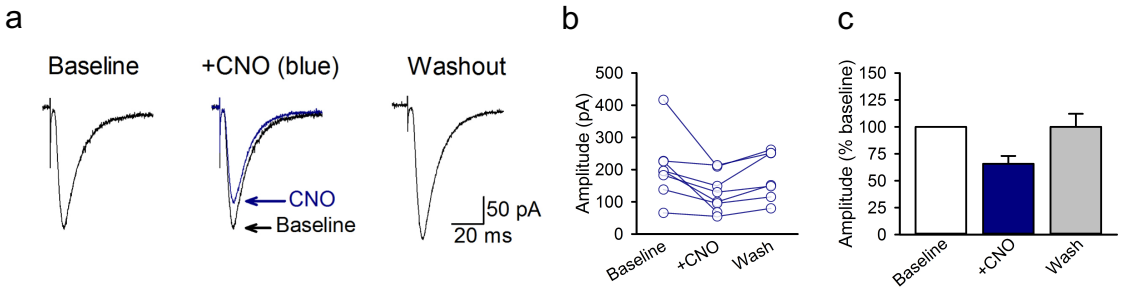
Chapter 2 Figure 6: Experiment 6: Selectivity of designer receptors for TH positive VTA neurons. **a** A representative coronal 4X optical plane through the VTA showing fluorescence indicative of mCherry (red; top; reporter for hM4Di; amplified with Alexa 594) and TH (green; bottom; Alexa 488), and a corresponding merged image at the right. **b** The number of cells counted in each 300 x 300 µm optical section. **c** Cell counts indicate the specificity of mCherry expression in TH+ neurons as well as the proportion of TH+ neurons that were transfected. **d** A representative area of a 40X image used for the analyses in **b** and **c**. Images showing DAPI, TH, mCherry and a merge of all three signals are shown. Cells with colocalized TH and mCherry signals all had a nucleus indicated by DAPI (blue). **e** A representative 4X confocal image through the striatum showing an injector tip in the NAc core (arrow) and fluorescence indicative of mCherry (red; reporter for hM4Di; amplified with Alexa 594), Tyrosine Hydroxylase (TH; green; Alexa 488), and the nuclear marker DAPI (blue). A 60X image showing **f** TH+, **g** mCherry+, and **h** merge signal from neural processes in the NAc core near the injector tip (arrow in **e**). **i** A representative 4X confocal image through the striatum showing an injector tip in the NAc shell (arrow) and fluorescence indicative of mCherry (red; reporter for hM4Di; amplified with Alexa 594), Tyrosine Hydroxylase (TH; green; Alexa 488), and the nuclear marker DAPI (blue). A 60X image showing **j** TH+, **k** mCherry+, and **l** merge signal from neural processes in the NAc shell near the injector tip (arrow in **i**). Images **f**-**h** and **j**-**k** in this figure are z-projections of maximum signal to highlight processes across the z-plane. Averaged data are mean ± s.e.m.

Figure 6

Experiment 7: Functional validation of circuit-specific chemogenetic inhibition

To validate the ability of CNO to inhibit terminals of VTA dopamine neurons expressing inhibitory designer receptors in the NAc, *in vitro* intracellular excitatory synaptic potentials in medium spiny neurons (MSNs) were recorded. Cells were identified as MSNs based on the characteristic slow, ramp-like depolarization of the membrane preceding spike firing[150,158] and the characteristic action potential waveform followed by a prominent fast afterhyperpolarization.

A 5 min bath application of 1 μM CNO to inhibit hM4Di-expressing terminals of VTA neurons within the NAc core did not produce significant changes in action potential number [2.4 ± 0.5 at baseline vs 1.7 ± 0.5 in CNO, $t_{(7)}$=1.18, p=.28], width [9.7 ± 0.7 ms at baseline vs 9.5 ± 0.7 ms in CNO, $t_{(7)}$=1.76, p=.14], or height [93.9 ± 3.4 mV at baseline vs 94.6 ± 3.9 mV in CNO, $t_{(7)}$=0.23, p=.83] during injection of positive current steps. However, CNO acting on inhibitory designer receptors on dopamine terminals significantly lowered action potential threshold from -43.2 ± 1.5 mV at baseline to -49.7 ± 2.4 in CNO [$t_{(7)}$=5.63, p=.002], which is consistent with the observation that dopamine agonists can shift threshold to more depolarized voltages[159]. CNO also resulted in a small decrease in the fast afterhyperpolarization [5.7 ± 0.7 mV at baseline to 4.6 ± 0.7 in CNO, $t_{(7)}$=3.52, p=.017]. Critically, application of CNO reduced the amplitude of excitatory postsynaptic currents (EPSCs) to 65.7 ± 7.3% of baseline levels from 206.1 ± 35.4 pA to 127.8 ± 21.3 pA [n=8; $F_{(2,13)}$=6.77, p=.01; Newman-Keuls p=.01; **Fig 7a-c**]. In two neurons, subsequent application of the AMPA/NMDA receptor blocker kynurenic acid (50 μM) completely blocked the EPSCs (data not shown), indicating that the synaptic responses were mediated by glutamatergic transmission. Thus, activation of inhibitory designer receptors in the terminals of VTA neurons in the NAc core inhibited excitatory postsynaptic responses in MSNs.



Chapter 2 Figure 7: Experiment 7: Functional validation of circuit-specific chemogenetic inhibition. **a** Example traces show averaged excitatory post synaptic currents (EPSCs) for a NAc core medium spiny neuron before (baseline), during (+CNO), and after (washout) 5 min application of 1 μM CNO to striatal slices containing hM4Di-expressing terminals from VTA dopamine neurons. **b** Peak amplitudes of EPSCs recorded from individual medium spiny neurons (MSNs) innervated by hM4Di-expressing dopaminergic terminals. **c** Normalized mean EPSC amplitudes for the same group of MSNs. Averaged data are mean ± s.e.m.

Figure 7

Discussion

An extensive literature implicates the dopamine system in alcohol use disorder and relapse, however there remains a lack of efficacious pharmacotherapies which may be attributed to a void in understanding the roles that separable dopaminergic circuits play in behaviour[160–162]. The principal objective of chapter 2 was to establish a validated circuit specific chemogenetic approach and uncover the roles that dopaminergic VTA-to-NAc core and shell projections play in responding to a discrete alcohol CS in different contexts. To this end, it was demonstrated that VTA dopamine neurons and their projection to the NAc core are necessary for responding to a discrete alcohol CS, whereas the dopaminergic projection to the NAc shell is necessary for the elevation of this behaviour in an alcohol context. This neural dissociation refines and advances our understanding of how the mesolimbic dopamine system regulates behavioural responses to discrete alcohol cues in different contexts.

First, pharmacology was used to implicate the dopamine system in responding to a discrete alcohol CS (Exp. 1). Interestingly, the systemic actions of both D1-like and D2-like receptors appeared necessary for general port directed behaviour, as both dopamine antagonists reduced NonCS port entries. However, CS responding required D2-like, but not D1-like receptors. While the reduction in general port directed behaviour may have been due in part to locomotor deficits caused by blocking dopamine receptors, this explanation is unlikely because the same dose of eticlopride used in the present study (10 μg/kg) had no impact on locomotor behaviour when directly tested in a separate study[163,164]. Further, CS port entries during a Pavlovian conditioning session in which the CS was paired with alcohol[106] were unaffected by 10 μg/kg eticlopride. Importantly, the results from experiment 1 implicate the dopamine system, particularly D2-like receptor activity, in responding to a discrete alcohol CS.

Using a chemogenetic approach in TH::Cre rats it was possible to selectively target VTA dopamine neurons, and examine their role in responding to a discrete alcohol CS. Designer receptors were expressed across the anterior-posterior and medio-lateral expanse of VTA dopamine neurons that project to both the NAc core and shell[68], with relatively minimal expression in the substantia nigra. The transfection was highly selective (95.8%) and restricted to a relatively small proportion of TH positive VTA neurons (24.8%; Exp. 6). Importantly, this chemogenetic approach was used to confirm that the dopamine system, particularly VTA dopamine neurons, was necessary for responding to a discrete alcohol CS (Exp. 2). Interestingly, targeting specifically VTA dopamine neurons circumvented the reductions in

general port-directed behaviour seen with dopamine antagonists, as NonCS port entries were unaffected by chemogenetic inhibition of VTA dopamine neurons. Importantly, neither the lowest effective CNO dose in this experiment nor a dose of clozapine that could have been produced through reverse metabolism had an effect on CS responding in rats that did not express designer receptors (Exp. 3). Thus, chemogenetic inhibition of VTA dopamine neurons reduced CS responding without affecting general port-directed behaviour, and this effect could not be explained by off-target effects of CNO or its parent-compound clozapine.

Focusing the chemogenetic approach on specific projections demonstrated that inhibition of VTA dopamine inputs to the NAc core reduced CS responding in both the alcohol and neutral context, which suggests that this projection is critical for responding to a discrete alcohol CS (Exp. 4). The exact nature of the involvement of the VTA-to-NAc core circuit in CS responding might arise from the NAc core being necessary for orchestrating behaviour in response to the best predictors of reinforcement. Supporting this idea, observational studies reveal the development of dopamine transients in the NAc core that are time locked to the onset and offset of food-predictive cues[165], and that track closely with the earliest reliable predictor of reinforcement[86]. Also, pairing a discrete CS with optogenetic stimulation of the VTA-to-NAc core projection is sufficient to produce a conditioned response; a finding that is not recapitulated with VTA-to-NAc shell stimulation[67]. The finding that CS responding was reduced by chemogenetic VTA-to-NAc core inhibition in both the alcohol and neutral context, strongly supports the hypothesis that this projection subserves responding to discrete alcohol-predictive cues.

Chemogenetic inhibition of VTA dopamine inputs to the NAc shell (Exp. 5) had no effect on CS responding in a neutral context (**Fig 5f** blue bars), but selectively reduced the elevation of this behaviour in the alcohol context (**Fig 5f** grey and black bars), which aligns with the hypothesis that NAc shell activity underpins the impact of context on drug-seeking behaviours[56,70,166–168]. One such study contributing to this hypothesis has shown that optogenetic inhibition of NAc shell MSNs projecting onto GABAergic interneurons in the VTA results in a net inhibition of VTA dopamine neurons, and this reduction in dopamine activity is associated with the attenuation of context-induced renewal of alcohol-seeking[166]. In the current study, chemogenetically silencing dopamine terminals in the NAc core reduced the strength of synaptic inputs onto MSNs (Exp. 7). A similar reduction in dopaminergic inputs to the NAc shell may have reduced the excitability of MSNs that project back onto GABA interneurons in the VTA[66,166,169], contributing to inhibition within the VTA that regulates the impact of alcohol

contexts on CS responding. Importantly, the finding that chemogenetic VTA-to-NAc shell inhibition reduced CS responding in the alcohol, but not neutral, context is compelling evidence that this projection supports the influence of context over responding to discrete alcohol-predictive cues.

The behavioural effects that were observed following circuit-specific chemogenetic manipulations are complimented by electrophysiological validation (Exp. 7). It had been shown previously that CNO acting on inhibitory designer receptors on VTA dopamine neurons decreases cellular excitability and evoked dopamine release[142], however it was unknown whether these effects impacted postsynaptic MSN activity. In the current experiment, CNO decreased the amplitude of evoked EPSCs onto MSNs innervated by VTA dopamine terminals expressing inhibitory designer receptors. This chemogenetic decrease in MSN EPSC amplitude, through actions of CNO on VTA dopamine terminals expressing designer receptors, is consistent with at least three mechanisms by which dopamine can influence the excitability of striatal MSNs. First, dopamine can act synergistically at D1 and D2 receptors on MSNs to enhance MSN activity[170,171]. Second, dopamine release from VTA terminals in the NAc can act on corticostriatal terminals to enhance glutamatergic input onto NAc MSNs[76,170,172–175], which would be decreased by chemogenetic inhibition of VTA dopamine terminals. Third, activation of inhibitory designer receptors could reduce EPSC amplitude by decreasing co-release of glutamate from VTA dopamine terminals[176–179]. The finding that chemogenetic inhibition of VTA dopamine terminals in the NAc core reduced the amplitude of MSN EPSCs was an important validation of the circuit-specific chemogenetic approach, and further, confirms that inhibitory designer receptors expressed on VTA terminals, when activated by CNO, affect postsynaptic MSN activity.

In summary, chapter two described the roles of midbrain dopamine neurons and their projections to the NAc core and shell in responding to a discrete alcohol CS in different contexts. Using pharmacology and chemogenetics it was demonstrated that VTA dopamine neurons are necessary for responding to a discrete alcohol CS. Further, through systematic manipulations of context combined with circuit-specific chemogenetic inhibition, it was shown that a VTA-to-NAc core dopamine circuit was necessary for responding to a discrete alcohol CS whereas a VTA-to-NAc shell dopamine circuit supported the elevation of this behaviour in an alcohol context. Importantly, the targeting of VTA dopamine neurons and the circuit-specific chemogenetic approach was validated using immunocytochemistry and electrophysiology,

which revealed that chemogenetic manipulations of VTA-to-NAc core projection neurons influenced the excitability of postsynaptic MSNs. Ultimately, an improved understanding of the neural substrates that control responding to discrete alcohol cues, and the influence of context over this behaviour, will inform the development of more precise interventions to prevent alcohol-seeking and relapse in humans.

Chapter 3: The role of dopaminergic and glutamatergic activity in responding to a discrete alcohol CS in different contexts

Introduction

There is evidence in this thesis and elsewhere that dopaminergic activity is necessary for responding to discrete alcohol cues[106] and the elevation of drug-seeking behaviour in drug-associated contexts[95], however, considerably less is known about the neural activity sufficient to elevate responding to discrete drug cues. Chapter two of this thesis presented compelling evidence that the dopaminergic projection from the VTA to the NAc shell was necessary for the elevation of responding to a discrete alcohol CS in an alcohol context. Others have also demonstrated the necessity of the NAc shell for responding to discrete drug cues in a drug-associated context[56,70,95]. If either the dopaminergic projection from the VTA-to-NAc shell or general activity in the NAc shell, play a causal role in the elevation of responding to a discrete alcohol CS, then increasing activity in this projection or subregion should elevate responding to a discrete alcohol CS. The Pavlovian conditioning with context alternation procedure is well-suited to address these predictions because the impact of neural manipulations on CS responding in the neutral context can be compared to responding in the alcohol context, thus assessing whether CS responding is elevated to levels akin to the alcohol context. The aim of chapter three was to investigate whether increasing activity in VTA dopamine neurons, their projections to the NAc shell, or general activation of the NAc shell, was sufficient to elevate responding to a discrete alcohol CS.

First, experiment 1 was conducted to test the prediction that increasing VTA dopaminergic activity would elevate CS responding in a neutral context. This prediction was supported by the findings that systemic administration of dopamine antagonists reduce responding to a discrete alcohol cue[85,106] and that contexts associated with alcohol, which potentiate responding to a discrete alcohol cue, evoke dopamine release in the NAc[79,85,180]. The necessity of dopaminergic activity for responding to a discrete alcohol cue, and the naturally occurring elevation in NAc dopamine levels in an alcohol-associated context suggest that elevating dopamine levels might be sufficient to drive responding to a discrete alcohol CS. Furthermore, systemically administered dopamine agonists can trigger the reinstatement of cocaine seeking[181] demonstrating that broad dopaminergic activity can elevate drug-seeking behaviour. Here, using an excitatory chemogenetic approach in TH::Cre rats, the sufficiency of VTA dopaminergic activity to elevate responding to an alcohol CS in a neutral context was tested.

Experiment 2 was conducted as a follow-up to experiment 1, to ensure that the excitatory chemogenetic approach was capable of affecting behaviour in a vetted protocol. It had been shown previously that chemogenetically activating VTA dopamine neurons suppressed feeding behaviour during the first two hours of food availability in a restricted feeding schedule[157]. Experiment 2 was designed to replicate this finding using the same rats from experiment 1, and to more comprehensively examine consummatory behaviour in the home-cage. Specifically, both feeding and drinking behaviour were measured, and consumption during both the first two hours and the entire duration of the feeding window, were analyzed. Also, the capacity for clozapine to act as an efficacious ligand for designer receptors was investigated in this protocol.

The involvement of neural substrates in behaviour is often projection specific[182], and experiment 3 was conducted to examine the sufficiency of the dopaminergic VTA-to-NAc shell projection to elevate responding to a discrete alcohol CS. An example of a projection-specific effect was reported in chapter 2 of this thesis; broad chemogenetic inhibition of VTA dopamine neurons reduced CS responding in a neutral context (Ch 2 Exp. 2), whereas selective inhibition of the dopaminergic VTA-to-NAc shell projection did not affect CS responding in a neutral context (Ch 2 Exp. 5). Specifically, experiment 3 was designed to test whether chemogenetic activation of the dopaminergic VTA-to-NAc shell projection was sufficient to elevate responding to a discrete alcohol CS in a neutral context to levels observed in an alcohol context. Based on the result that the dopaminergic VTA-to-NAc shell projection was necessary for the elevation of CS responding in an alcohol context, it was reasoned that activity in the VTA-to-NAc shell projection might be recruited by the alcohol context. Thus, it was predicted that artificially activating the dopaminergic VTA-to-NAc shell projection using chemogenetics would recapitulate the necessary neural signals to elevate CS responding in the neutral context, as if the rats were in the alcohol context. The rational for this experiment is supported by the findings that glutamatergic inputs to the NAc shell, which are modulated by dopamine[183,184], and dopamine neurotransmission are necessary for the context renewal of alcohol-seeking[70,71]. However, it is not known whether increasing activity in projections to the NAc shell is sufficient to elevate responding for alcohol. Interestingly, dopamine microinfused directly into the NAc (core and shell not separated) triggers the reinstatement of cocaine-seeking, which may suggest that activation of dopaminergic inputs to the NAc shell could elevate responding to an alcohol CS[185].

Experiment 4 was conducted to validate the excitatory (hM3Dq) circuit-specific chemogenetic approach used in experiment 3. When activated by CNO, excitatory designer receptors have been shown to induce c-fos expression, increase the spontaneous firing rate, and depolarize VTA dopamine neurons[134,143]. Importantly, excitatory designer receptors are trafficked to the terminals of VTA dopamine neurons[134,143,144], and evoke dopamine release in the NAc when bound by CNO[134], which support the use of these receptors in a circuit-specific manner. However, no study has examined whether the activation of excitatory designer receptors on VTA dopamine terminals in the NAc serves to influence the activity of postsynaptic MSNs. In experiment 4, electrically-evoked EPSCs were recorded from NAc core MSNs that were innervated by VTA dopamine terminals expressing excitatory designer receptors, to validate the excitatory circuit-specific chemogenetic approach.

Glutamatergic and dopaminergic signalling in the NAc have been implicated in animal models of drug-seeking behaviour[186]. Interestingly, glutamate and dopamine levels in the NAc are interdependent – glutamate analogues microinfused into the NAc produce dopamine release, and the converse[75–78]. In particular α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) which binds to the AMPA glutamate receptor, is capable of facilitating or reinstating cocaine self-administration when microinfused into the NAc (shell and core not separated)[185,187]. Furthermore, glutamatergic inputs to the NAc shell from the ventral subiculum (vSub) are necessary for the context renewal of alcohol-seeking in a model of relapse that used punishment to reduce operant responding[70]. While general activity in the NAc shell appears to be necessary for alcohol context renewal[56] and the elevation of responding to a discrete alcohol CS in an alcohol context[73], it is unknown whether AMPA in the NAc shell can augment responding to a discrete alcohol CS. Experiment 5 was conducted to test whether microinfusing AMPA in the NAc shell, and consequently increasing NAc shell activity, impacts responding to a discrete alcohol CS in an alcohol or neutral context.

The overall aim of chapter 3 was to examine the sufficiency of VTA dopamine neurons, their projections to the NAc shell, and glutamatergic activity in the NAc shell to elevate responding to a discrete alcohol CS in a neutral context, to levels comparable to those observed in an alcohol context. First, a chemogenetic approach in TH::Cre rats was used to activate VTA dopamine neurons during a test for CS responding in a neutral context (Exp. 1). To validate that this chemogenetic approach had the capacity to affect behaviour, a thorough investigation of feeding and drinking behaviour was conducted (Exp. 2). Next, the potential for circuit-specific

dopaminergic activity to elevate responding to a discrete alcohol CS was examined by chemogenetically activating the dopaminergic VTA-to-NAc shell projection (Exp. 3). Importantly, the excitatory circuit-specific chemogenetic approach was validated using electrophysiology (Exp. 4). Lastly, the potential for increased glutamatergic activity within the NAc shell to impact responding to a discrete alcohol CS in different contexts was examined (Exp. 5).

Methods

Subjects

Male, Long-Evans, TH::Cre$^{+/-}$ rats (n=33) were bred in-house on a mixed Charles River and INVIGO background and used in experiments 1 (n=7), 2 (n=7 from Exp. 1; n=13 from Ch 2 Exp. 3), 3 (n=9), 4 (n=5). Male, Long-Evans rats (n=13) were ordered from INVIGO Laboratories, Indianapolis, USA and weighed 220-275 g on arrival. All rats were individually housed in standard polycarbonate shoebox cages (20 x 24 x 45 cm) and maintained on a 12 h light-dark cycle (lights on at 0700) at 21±2°C at 40-50% humidity. All procedures were conducted in the light phase and rats had unrestricted access to chow (Charles River Rodent Diet #5075), tap water, and a nylabone^{TM} chew-toy except experiment 2 wherein rats temporarily received mild food restriction. All experimental procedures complied with the Animal Research Ethics Committee at Concordia University and the Canadian Council on Animal Care regulations.

In experiment 3, half of the rats received a control virus infused into their ventral subiculum (vSub) to examine whether two viral constructs could be expressed without impacting behaviour. A viral construct encoding the fluorescent protein EYFP under the control of a general neuronal promotor[188] (CamKII) was used so that vSub and designer-receptor expressing VTA cells (mCherry) could be imaged with separate channels. The factor group was included in all preliminary analyses for experiment 3 but failed to reveal any significant group effects or interactions with group and as such the factor group was not included in the analyses reported here. In total, 4 rats failed to express designer receptors in the VTA, 1 rat failed to acquire Pavlovian conditioning, 1 rat had damage from an infection in the location where microinfusions occurred, 3 rats had missed placement, and 1 rat was accidentally given extinction sessions instead of retraining sessions between tests. Data from these rats was excluded from all analyses and graphs.

Behavioral apparatus

Behavioral training and testing occurred in 12 conditioning chambers (ENV-009A; Med-Associates Inc.), enclosed in fan-ventilated (~77 dB) sound-attenuating, melamine cubicles (53.6 x 68.2 x 62.8 cm). The right wall featured a fluid port (17.5 cm from rear wall, 9 cm from front door) that contained two wells (ENV-200R3AM). Fluid delivery into one well occurred through a 20 ml syringe attached to a pump (PHM-100, 3.33 rpm) located outside the cubicle. Fluid port entries were measured with an infrared beam (ENV-205M) and recorded to a computer using Med PC-IV software, which also controlled fluid delivery and stimulus presentations. The upper left wall featured a clicker stimulus (ENV-135M, 8 dB above background), a continuous white noise stimulus generator (ENV-225SM, 8 dB above background), and a white house-light (ENV-215M).

Solutions and reagents

Odours were prepared by adding lemon oil (SAFC Supply Solutions, St-Louis, MO, USA, #W262528) or benzaldehyde (almond odor, OMEGA Chemical Company Inc., Levis, QC, Canada, #B37-50) to tap water (10%, v/v). Alcohol (15 % ethanol, v/v) was prepared every week by diluting 95% ethanol in tap water (room temperature). CNO (Clozapine-*n*-oxide, Tocris #4936 or NIMH C-929) for systemic administration was dissolved in 5% dimethyl sulfoxide (DMSO) and 95% sterile 0.9% saline to make a 10 mg/ml concentration. Clozapine (AdooQ, #A10236-500) was dissolved in 5% dimethyl sulfoxide and 95% sterile 0.9% saline to make a 0.1 mg/ml solution. CNO (abcam, #ab141704) was dissolved in sterile 0.9% saline (3 mM for intracerebral microinfusions) or artificial cerebrospinal fluid (1 μM for *in vitro* electrophysiology). α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; Tocris, 1074) was dissolved in sterile saline (0.1 mM) for intracerebral microinfusion. Viral vectors were bought from the University of North Carolina Vector Core [AAV8-hSyn-DIO-hM3D(Gq)-mCherry (titer $5.9x10^{12}$)] or Addgene [AAV8-hSyn-DIO-mCherry (titer $2.1x10^{13}$), AAV8-hSyn-DIO-hM3D(Gq)-mCherry (titer 2.2×10^{13}), AAV2-CamKII-EYFP (titer 2×10^{12})].

Surgery

Anesthetized (isoflurane, 5% induction, 2-3% maintenance) rats were secured in a stereotaxic frame and administered atropine (0.1 ml/kg) subcutaneously (s.c.). Bilateral, ventral tegmental area (VTA) microinfusions of 1 μl (0.1 μl/min, 10 min diffusion) of AAV8-hSyn-DIO-mCherry, or AAV8-hSyn-DIO-hM3D(Gq)-mCherry viral vectors were made through a 26 gauge injector connected with PE20 tubing to a Hamilton microinjection syringe on a Harvard

Apparatus, Pump 11 Elite. VTA coordinates (in mm) from bregma were: AP -5.5, ML ±1.84 (with 10° angle), DV -8.33. Bilateral, ventral subiculum (vSub) microinfusions of 1 μl (0.25, μl/min, 1 min diffusion)[70] of a control viral construct (AAV2-CamKII-EYFP) were made through a 26 gauge injector connected with PE20 tubing to a Hamilton microinjection syringe on a Harvard Apparatus, Pump 11 Elite. vSub coordinates (in mm) from bregma were: AP -6.0, ML ±5.4 (4° angle), DV -8.62.

Where appropriate, 26-gauge bilateral guide cannulae (PlasticsOne, C315G-SPC) were implanted 3 mm dorsal to the microinjection site using the following coordinates (in mm) at a 10° angle: NAc shell AP +1.68, ML ± 2.23, -7.35. After surgery, rats received buprenorphine (0.1 mg/kg, s.c.) and ≥7 days to recover.

General behavioural procedures

Home-cage alcohol exposure

Imposing intermittent schedules of alcohol availability has been shown to produce gradual elevations in ethanol consumption[106,114–119]. All rats received 24 h access to alcohol and tap water every other day for 12 sessions, over 24 days. On the intervening days only, water was available. Alcohol was provided in a 100 ml graduated cylinder fitted with a rubber stopper containing a sipper tube with a metal ball bearing to minimize spillage. Alcohol cylinders and water bottles were placed onto opposite sides of a standard cage lid and weighed before and after every 24 h session. The position of the water bottle and alcohol cylinder was switched at the beginning of every session to control side-preferences. A filled alcohol cylinder and water bottle were placed onto two empty cages and weighed to monitor spillage. The average spillage as a result of handling the cylinder and bottle was subtracted from the rats daily consumption. Rats were weighed every other day before receiving alcohol cylinders. Home-cage alcohol exposure was conducted identically for all experiments in this chapter except for experiment 4, as rats used in electrophysiological experiments were not exposed to alcohol.

Habituation

On the last day of home-cage alcohol exposure, rats were brought to the behavioural testing room in their home-cages and individually handled. On the two subsequent days all rats were habituated to Context 1 and then 24 h later to Context 2 in the conditioning chambers. Context 1 consisted of black walls, a clear Plexiglas floor, and a lemon odour. Context 2 consisted of clear Plexiglas walls, a wire-mesh floor, and an almond odour. Odours (3 sprays)

were applied to a petri dish placed in the waste-pan under the chamber floor. Entries into a fluid port in the conditioning chamber were recorded during each context habituation session. All habituation sessions were 20 min long. Behaviour room and conditioning chamber habituation sessions occurred at the same time of day as subsequent training and test sessions.

Pavlovian conditioning with context alternation

Rats were assigned to context 1 or context 2 for Pavlovian conditioning ('alcohol context'), while the other context served as the 'neutral context'. Discrete stimuli were a 10 s, continuous white noise or 10 s clicker (5 Hz). One stimulus (conditioned stimulus, CS) was paired with alcohol delivery in the alcohol context and the other (neutral stimulus, NS) was presented without alcohol in the neutral context. Rats were counterbalanced across contexts, stimulus identity, and session order such that there were no differences in home-cage alcohol consumption. Rats then received one training session per day (73.5 min) that alternated between each context until 12 sessions of Pavlovian conditioning in the alcohol context and 12 sessions of exposure to the NS in the neutral context had occurred.

During training sessions, rats received 15 stimulus presentations (either CS or NS as per the appropriate context) with intervals of 140, 260, or 380 s between trials [mean inter-trial interval (ITI) = 260 s]. In the alcohol context, CS presentations co-terminated with 6 s of syringe pump operation to deliver 0.2 ml of alcohol into the fluid port. In the neutral context, NS presentations also co-terminated with 6 s of syringe pump operation, but no alcohol was delivered. The protocol for Pavlovian conditioning with context alternation was conducted identically in all experiments in this chapter except for experiment 4 as the electrophysiology experiment used rats that had not undergone Pavlovian conditioning.

Experiment 1. Effect of chemogenetic excitation of VTA dopamine neurons on CS responding

On the last two sessions of Pavlovian conditioning with context alternation, TH::Cre rats expressing the excitatory designer receptor (hM3Dq) in the VTA (n=7) received two intraperitoneal (i.p.) 1 ml/kg saline injections 30 min before being placed in the conditioning chamber outfitted as the alcohol or neutral context. Next, three CS responding tests in the neutral context were conducted after rats received systemic i.p. injections of vehicle, CNO at 10 mg/kg, or clozapine at 0.1 mg/kg[131,155]. Tests were separated by 2 retraining sessions, alternating between the alcohol and neutral contexts (one session per context).

Experiment 2. Effect of chemogenetic excitation of VTA dopamine neurons on feeding behaviour

Approximately, 12 weeks after the completion of experiment 1, the rats from this experiment that expressed excitatory designer receptors on VTA dopamine neurons (n=7) and a group of TH::Cre rats that expressed a control fluorescent protein on VTA dopamine neurons (n=13; same rats from Ch 3 Exp. 3) were placed on a restricted feeding schedule[157]. A fixed amount of food (~ 60 g) was placed on the home-cage food hopper every day at 7:00 pm which aligned with the onset of the dark cycle and the natural onset of feeding behaviour in rats[189]. Food was removed and weighed every 30 minutes for the first two hours of food access (i.e. until 9:00 pm). Food was weighed again at 11:00 am the next day and removed. Water was continuously available through water bottles that were placed on the home-cage which were weighed at the same time as food. Weighing food and water for all the rats could be conducted in less than three minutes. In total, the restricted feeding schedule was maintained for ten days. After a baseline of three days of restricted feeding, the first of three test was conducted where rats received i.p. injections of vehicle, CNO (10 mg/kg), or clozapine (0.1 mg/kg) 30 min before food was made available and the onset of the dark cycle, according to a within-subjects, latin-square design.

Experiment 3: Chemogenetic excitation of a dopaminergic VTA-to-NAc shell circuit

On the last two sessions of Pavlovian conditioning with context alternation, TH::Cre rats expressing excitatory designer receptors in the VTA (*n*=9) received a saline habituation microinfusion (0.15 μl over 1 min) into the NAc shell before a training session in the alcohol and neutral context. At 24 h after the last training session, the first of four tests for CS responding was conducted. A similar number of rats were tested in the alcohol or neutral context after receiving a vehicle or CNO (3 mM)[146,147] microinfusion (0.3 μl over 1 min) 5-15 min before the test session. One retraining session in the alcohol context and one in the neutral context separated each of the four tests.

Experiment 4. Functional validation of circuit-specific chemogenetic excitation

Male, TH::Cre rats (n=5) received stereotaxic surgery to deliver VTA microinfusions of 1 μl of AAV8-hSyn-DIO-hM3D(Gq)-mCherry. At 4-6 weeks later, rats were anaesthetized with isoflurane and decapitated. Brains were rapidly extracted and submerged in an ice-cold HEPES-based artificial cerebrospinal fluid (ASCF) solution containing (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 20 HEPES, 30 $NaHCO_3$, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3

sodium pyruvate, 12 N-acetyl-L-cysteine (NAC), 10 $MgSO_4$, and 0.5 $CaCl_2$ (pH adjusted to ≈7.3-7.4 using 10 M NaOH) saturated with 95% O_2/5% CO_2. Coronal slices (300 μm) containing the NAc were obtained using a vibratome (Leica, VT1200) and transferred to a warm (34˚C), high-choline incubation solution containing (in mM): 92 choline chloride, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 12 NAC, 10 $MgSO_4$, and 0.5 $CaCl_2$, where they recovered for 12 min. Subsequently, slices were incubated at room temperature, in a normal ACSF solution containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 dextrose and were allowed to recover for a minimum of 1 h prior to experiments. Once transferred into the recording chamber, slices were perfused with normal ACSF at 2 ml/min and were visualized using an upright fluorescence microscope with a 40X water-immersion objective, differential interference contrast optics (Olympus, BX51WI), and XM-10 monochrome camera for viewing (Olympus, CellSens V1.8). mCherry fluorescence in the NAc core was verified at 4X and 40X magnification prior to recordings.

Whole-cell patch-clamp pipettes made from boroscillate glass (1.0 mm OD, 3-5 MΩ) were filled with a recording solution containing (in mM): 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 2 ATP-tris, 0.4 GTP-tris (pH adjusted to 7.25 using KOH, 270-280 mOsm) and were lowered onto visually-identified neurons in the NAc core. Tight seals were obtained (1.3-6.6 GΩ), and cells were allowed to stabilize in whole-cell configuration for 10 min prior to recordings. Recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices), digitized (Digidata 1440A, Molecular Devices), and were stored using pClamp 10.3 software (Molecular Devices). Access resistance was 19.9 ± 2.2 MΩ, and series resistance was uncompensated. All cells recorded had a resting membrane potential below -65 mV. Cellular input resistance, membrane capacitance, and access resistance were monitored during each recording condition.

Cells were initially selected based on visual criteria; medium spiny neurons and GABAergic interneurons possess smaller soma in comparison to cholinergic interneurons (8-20 μm vs 20-50 μm[149–151]) and any cells with soma >30 μm were not recorded from. GABAergic interneurons and medium spiny projection (MSN) neurons of the accumbens core were differentiated electrophysiologically by injecting 500 ms hyperpolarizing and depolarizing current steps between -100 and 100 pA in 10 pA intervals from the holding potential of -70mV. Peak input resistance was measured at the largest voltage change in response to a -100 pA pulse,

and steady state input resistance was assessed just prior to the end of the current step. Action potential properties were measured from the first action potential evoked in response to positive current injection.

Synaptic responses were evoked using a bipolar stimulating electrode made from two tungsten electrodes (≈1 MΩ, FHC Inc.) placed approximately 30 μm from the recording electrode. Evoked AMPA-receptor-mediated excitatory postsynaptic currents (EPCSs) were recorded at -70 mV, near the resting membrane potential of MSNs of the NAc core [152] using constant current stimulation pulses. For each cell, at least 10 consecutive synaptic responses free from artifacts or action potentials were averaged for each phase of the recordings. Dopamine release from VTA terminals to the NAc core is under tonic inhibition from aspiny GABAergic interneurons[153], and dopamine can also modify inhibition in the accumbens[152,154]. Because GABA neurotransmission can alter the excitability of medium spiny neurons, picrotoxin (50 μM) was included in the ACSF to block $GABA_A$-mediated inhibition and better assess the effects of CNO on VTA inputs to medium spiny neurons. Recordings were obtained before and after 10 min application of 1 μM CNO, and were also obtained after 20 min washout of CNO in the continued presence of picrotoxin. The amplitudes of averaged synaptic currents were measured using Clampfit 8.2 software (Molecular Devices) and normalized to the amplitude of responses recorded prior to CNO application.

Experiment 5. Effect of excitation of the NAc shell with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid on CS responding

On the last two sessions of Pavlovian conditioning with context alternation, wild-type rats (n=13) received a saline habituation microinfusion (0.15 μl over 1 min) into the NAc shell before a training session in the alcohol and neutral context. At 24 h after the last training session, the first of four tests for CS responding was conducted. A similar number of rats were tested in the alcohol and neutral context after receiving a vehicle or AMPA (0.1 mM) microinfusion (0.3 μl over 1 min) 5-15 min before the test session[109]. One retraining session in the alcohol context and one in the neutral context separated each of the four tests.

Histology and imaging

Rats from experiments 1-3 and 5 were deeply anesthetized with euthanyl™ (sodium pentabarbitol, 240 mg/kg, i. p.) and perfused with PBS (0.02 M, 250 ml, pH 7.2) and 4% paraformaldehyde in 0.02 M PBS (150 ml, pH 7.2). Brains were immediately removed,

cryoprotected a 4% paraformaldehyde 30% sucrose solution (50 ml, ~2-3 days), and then stored at -80°C until they were sectioned (40 μm thick) using a cryostat at -20°C. Nissl staining was conducted to assess histological placements of injector tips in the NAc shell. Unamplified mCherry signal was used to verify successful designer receptor expression by letting slides dry for 24 h after removal from the -20°C freezer and then coverslipping them with vectasheild™. mCherry fluorescence was assessed using a Leica DM4000B epifluorescence microscope at 5X and 10X magnification.

Analyses and Statistics

Software

Statistical analyses were conducted with SigmaPlot™ v12 or SPSS™ v20 and graphs were made with SigmaPlot™ v12.

Dependent variables

During home-cage alcohol consumption grams of ethanol consumed per kilograms of body weight (g/kg) was measured. In Pavlovian conditioning and test sessions the following variables were measured, the number of: port entries per session (total port entries), port entries during the 10 s CS (CS port entries), port entries made between CS offset and the next CS onset (140, 260, or 380 s; NonCS port entries), and port entries in the 10 s preceding the CS (PreCS port entries).

Analyses

Data from all experiments were analyzed using a repeated-measures (RM) ANOVA, and followed-up with post-hoc Bonferroni-corrected t-tests, or Newman-Keuls multiple comparisons. A Huynh-Feldt correction was applied when sphericity was violated in these analyses. All analyses used an alpha level of p=0.05.

Results

Experiment 1. Effect of chemogenetic excitation of VTA dopamine neurons on CS responding

To evaluate the impact of increasing activity in a subset of ventral tegmental area (VTA) dopamine neurons on responding to a discrete alcohol CS a chemogenetic approach in transgenic rats was used. Naïve TH::Cre male rats (n=7) were microinfused bilaterally into the VTA with the double-floxed[132] excitatory designer receptor construct[130] AAV8-hSyn-DIO-hM3Dq-

mCherry, resulting in the selective expression of the excitatory designer receptor (hM3Dq) in VTA dopamine neurons. This receptor induces neuronal burst firing when bound by the exogenous ligand clozapine-*n*-oxide (CNO)[130,131].

After recovering from surgery, rats received twelve sessions of exposure to alcohol in the home-cage (**Fig 1a**). The dependent measure for home-cage alcohol exposure was grams of ethanol consumed as a function of rat weight in kilograms (g/kg). Alcohol consumption was analyzed using a within-subjects RM ANOVA including the factor Session (1, 2, 3…12). Alcohol consumption increased across home-cage sessions [Session, $F_{(11, 66)}$=5.174, p<.001; **Fig 1b**].

Then, rats underwent Pavlovian conditioning with context alternation (**Fig 1c**). Port entries during training were analyzed using a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS, CS/NS) RM ANOVA. Port entries were elevated in the alcohol context and increased across sessions [Session, $F_{(11, 66)}$=5.459, p<.001; Context, $F_{(1, 6)}$=30.666, p=.001; Context x Session, $F_{(11, 66)}$=3.980, p<.001]. The elevation in port entries in the alcohol context was driven by CS port entries which increased into a plateau, whereas port entries during the PreCS in the alcohol context remained stably low [Interval, $F_{(1, 6)}$=25.237, p=.002; Interval x Session, $F_{(11, 66)}$=4.987, p<.001]. Port entries during the NS and PreNS intervals in the neutral context all remained stably low throughout conditioning relative to CS port entries in the alcohol context [Context x Interval, $F_{(1, 6)}$=29.625, p=.002]. Importantly, the increase in CS relative to PreCS port entries in the alcohol context, while port entries during all intervals in the neutral context remained low and stable, suggests that rats specifically acquired responding to the CS in the alcohol context [Context x Interval x Session, $F_{(11, 66)}$=3.954, p<.001; **Fig 1d** (left)].

The total number of port entries made during each training session was analyzed using a RM ANOVA including the factors Session (1, 2, 3, …12) and Context (Alcohol, Neutral). In the alcohol context, the total number of port entries were elevated relative to the neutral context [Context, $F_{(1, 6)}$=30.547, p=.001] wherein port entries declined across sessions [Session, $F_{(11, 66)}$=5.978, p<.001; Context x Session, $F_{(11, 66)}$=4.251, p<.001; **Fig 1d** (right)].

Next, CS responding was tested by presenting the CS without alcohol in the neutral context 30 min after i.p. injection of vehicle (5% DMSO in saline), CNO (10 mg/kg), or Clozapine (0.1 mg/kg; **Fig 1e**)[131,155]. Test data were analyzed with an Interval (PreCS, CS) by Treatment (Vehicle, CNO 10 mg/kg, Clozapine 0.1 mg/kg) RM ANOVA and NonCS port entries were

analyzed using a separate RM ANOVA including only the factor Treatment (Vehicle, CNO 10 mg/kg, Clozapine 0.1 mg/kg). PreCS port entries were minimal at test compared to CS port entries [Interval, $F_{(1, 6)}$=15.394, p=.008] and both were unaffected by CNO or clozapine [Treatment, $F_{(2, 12)}$=.062, p=.940; Treatment x Interval, $F_{(2, 12)}$=.299, p=.747; **Fig 1f** (left)]. NonCS port entries were similarly unaffected by CNO or clozapine [Treatment, $F_{(2, 12)}$=3.755, p=.054; **Fig 1f** (right)].

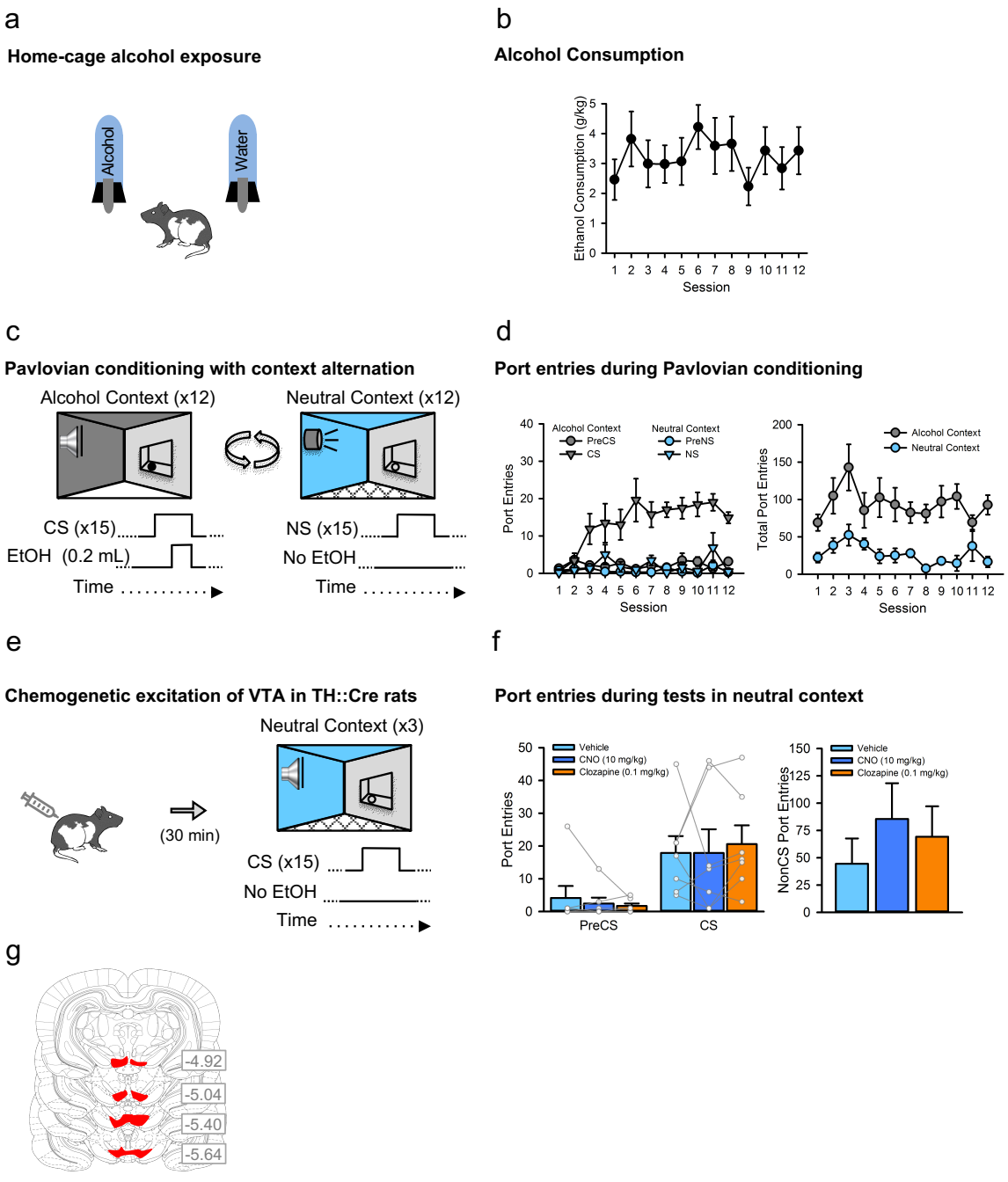

a
Home-cage alcohol exposure
Alcohol
Water
b
Alcohol Consumption
Ethanol Consumption (g/kg)
Session
c
Pavlovian conditioning with context alternation
Alcohol Context (x12)
Neutral Context (x12)
CS (x15)
EtOH (0.2 mL)
Time
NS (x15)
No EtOH
Time
d
Port entries during Pavlovian conditioning
Alcohol Context
Neutral Context
PreCS
CS
PreNS
NS
Port Entries
Session
Total Port Entries
e
Chemogenetic excitation of VTA in TH::Cre rats
Neutral Context (x3)
(30 min)
CS (x15)
No EtOH
Time
f
Port entries during tests in neutral context
Vehicle
CNO (10 mg/kg)
Clozapine (0.1 mg/kg)
Port Entries
PreCS
CS
NonCS Port Entries
g
-4.92
-5.04
-5.40
-5.64

Figure 1

Chapter 3 Figure 1: Experiment 1. Effect of chemogenetic excitation of VTA dopamine neurons on CS responding. **a** After recovering from surgery wherein TH::Cre rats (n=7) received microinfusion of a viral construct encoding for the excitatory designer receptor (AAV8-hSyn-DIO-hM3Dq-mCherry) in the ventral tegmental area (VTA), rats received 24 h access to a 15% ethanol (alcohol) solution in their home-cage every other day for a total of 12 sessions. Water and standard rat chow were continuously available. **b** Alcohol consumption shown as grams of ethanol over kilograms of body weight across 12 home-cage alcohol exposure sessions. **c** Then, rats received 12 Pavlovian conditioning sessions every other day in a distinct alcohol context wherein a discrete, 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (12 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. **d** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral context across sessions of Pavlovian conditioning with context alternation (right). **e** After Pavlovian conditioning with context alternation, rats received the first of 3 tests for CS responding in the neutral context which were conducted on separate days and intervened by retraining sessions. Before tests, rats received an intraperitoneal injection of vehicle, CNO (10 mg/kg), or clozapine (0.1 mg/kg). **f** PreCS and CS port entries (left) and NonCS port entries (right) during tests in the neutral context. **g** Maximal mCherry expression in the VTA for each rat is shown in schematics from the atlas of Paxinos & Watson (2008). Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 1

Experiment 2. Effect of chemogenetic excitation of VTA dopamine neurons on feeding behaviour

The lack of an effect of chemogenetic activation of VTA dopamine neurons on responding to a discrete alcohol CS may indicate that the chemogenetic technique was ineffective at manipulating neuronal activity, and consequently affecting behaviour. To evaluate this possibility, the effect of chemogenetic activation of VTA dopamine neurons on feeding behaviour was assessed using an assay that was previously shown to be sensitive to this manipulation (**Fig 2a**)[157]. Briefly, one group of rats expressing the excitatory designer receptor on VTA dopamine neurons (n=7, from Ch 3 Exp. 1) and another group of rats expressing a control fluorescent protein on VTA dopamine neurons (n=13, from Ch 2 Exp. 3) were mildly food restricted for the eight hours before lights were turned off and food was made available for the next 16 h. On three separate days, injections of vehicle, CNO (10 mg/kg), or clozapine (0.1 mg/kg) were administered 30 minutes before food access.

A previous study using this procedure reported that VTA dopamine activity reduced food consumption in the first two hours of food access and a similar analysis was performed in the current experiment. Additionally, food consumption during the entire 16 h feeding window was analyzed, as was water consumption because food and water consumption often affect one another[190]. Food and water consumption during the first two hours of food access and the entire 16 h window of food access was analyzed in four separate RM ANOVAs with the factors Treatment (Vehicle, CNO, Clozapine) and Group (hM3Dq, mCherry).

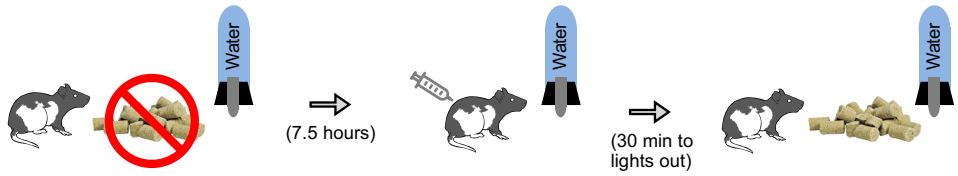
Food consumption during the first two hours of food access was significantly reduced after treatment with CNO or clozapine in rats expressing excitatory hM3Dq receptors, but not in rats expressing the control fluorescent protein mCherry, on VTA dopamine neurons [Treatment, $F_{(2, 34)}$=4.989, p=.013; Treatment x Group, $F_{(2, 34)}$=5.276, p=.01; Group, $F_{(1, 17)}$=2.455, p=.136; **Fig 2b**]. Bonferroni-corrected t-tests revealed that the hM3Dq and mCherry groups differed specifically in terms of their food consumption after receiving CNO [$t_{(17)}$=-2.478, p=.024] and clozapine [$t_{(17)}$=-2.409, p=.028] but not vehicle [$t_{(17)}$=.637, p=.532]. Interestingly, the reduction in food consumption during the first two hours of food access, was not recovered during the remainder of the 16 h feeding window as total food consumption was also reduced following CNO or clozapine treatment [Treatment, $F_{(2, 34)}$=.268, p=.767; Treatment x Group, $F_{(2, 34)}$=4.390, p=.02; Group, $F_{(1, 17)}$=2.026, p=.173; **Fig 2c**]. Bonferroni-corrected t-tests confirmed that the hM3Dq and mCherry groups differed in terms of total food consumption following CNO

treatment [$t_{(17)}$=-2.443, p=.026] and not vehicle [$t_{(17)}$=.932, p=.364] however, the comparison after clozapine treatment did not reach significance [$t_{(17)}$=-697, p=.108]

Water consumption was unaffected by treatment with CNO or clozapine during the first two hours of food access [Treatment, $F_{(2, 34)}$=.072, p=.931; Treatment x Group, $F_{(2, 34)}$=.210 p=.812; Group, $F_{(1, 17)}$=.133, p=.720; **Fig 2d**] and the entire 16 h feeding window [Treatment, $F_{(2, 34)}$=.927, p=.406; Treatment x Group, $F_{(2, 34)}$=.850, p=.436; Group, $F_{(1, 17)}$=1.927, p=.183; **Fig 2e**] in both groups of rats.

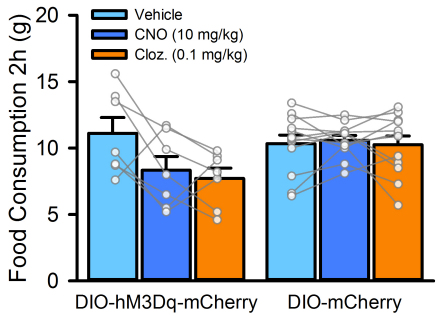
a

Effect of chemogenetic VTA excitation on home-cage food and water consumption under mild food restriction



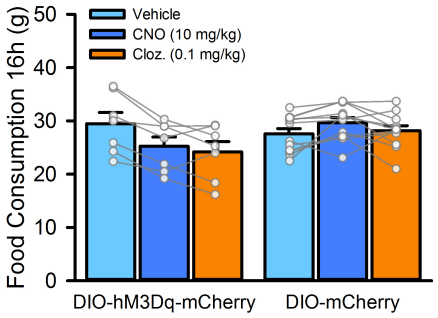
b

Food consumption during the first two hours of food access



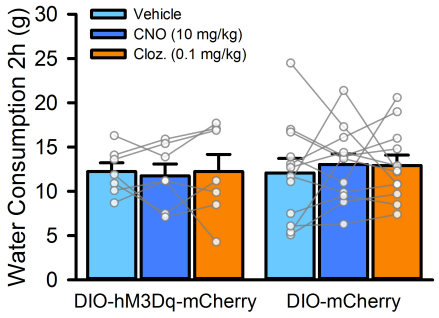
c

Food consumption during the 16h feeding window



d

Water consumption during the first two hours of food access



e

Water consumption during the 16h feeding window

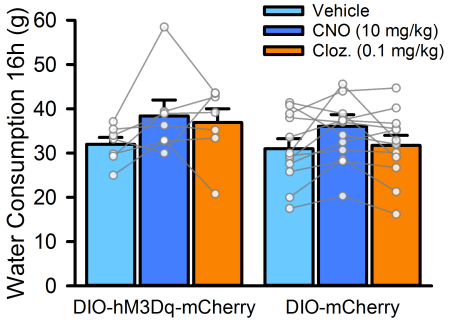


Figure 2

Chapter 3 figure 2: Experiment 2. Effect of chemogenetic excitation of VTA dopamine neurons on feeding behaviour. **a** Two groups of TH::Cre rats expressing either the excitatory designer receptor (n=7; DIO-hM3Dq-mCherry) or a control fluorescent (n=13; DIO-mCherry) in the VTA were placed on a restricted feeding schedule where water was continuously available but food was only available for 16 hours beginning with the onset of the dark cycle. Rats received an intraperitoneal injection of vehicle, CNO (10 mg/kg), or clozapine (0.1 mg/kg) 30 min before tests for food and water consumption. Tests were intervened by 2 days of restricted feeding when no injections were given. Food and water consumption during the first two hours of food access and the entire 16 h feeding window were measured. **b** Grams of food consumed during the first two hours of food access and **c** the entire feeding window. **d** Grams of water consumed during the first two hours of food access and **e** the entire feeding window. Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 2

Experiment 3. Chemogenetic excitation of a dopaminergic VTA-to-NAc shell circuit

A circuit-specific chemogenetic approach was used to test the prediction that activation of a dopaminergic projection from the VTA to the NAc shell was sufficient for the elevation of CS responding in a neutral context. The dopaminergic VTA-to-NAc shell projection was targeted by microinfusing the viral AAV8-hSyn-DIO-hM3Dq-mCherry construct into the VTA of naïve TH::Cre rats (n=9) and implanting bilateral cannulae above the NAc shell. The dopaminergic VTA-to-NAc shell projection could then be excited by microinfusing CNO through injectors targeting the NAc shell.

After recovering from surgery, rats received twelve sessions of exposure to alcohol in the home-cage (**Fig 3a**). The dependent measure for home-cage alcohol exposure was grams of ethanol consumed as a function of rat weight in kilograms (g/kg). Alcohol consumption was analyzed in a within-subjects RM ANOVA including the factor Session (1, 2, 3…12). Alcohol consumption increased across home-cage sessions [Session, $F_{(11, 88)}$=1.681, p=.091; **Fig 3b**].

Then, rats underwent Pavlovian conditioning with context alternation (**Fig 3c**). Port entries that occurred during the PreCS and CS intervals in the alcohol context, and the corresponding intervals in the neutral context were analyzed in a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS or CS/NS) RM ANOVA. Port entries in the alcohol context increased across sessions relative to the neutral context [Session, $F_{(11, 88)}$=4.799, p<.001; Context, $F_{(1, 8)}$=15.602, p=.004; Context x Session, $F_{(11, 88)}$=4.957, p<.001]. The elevation in port entries in the alcohol context was driven by CS port entries which increased into a plateau, whereas port entries during the PreCS in the alcohol context remained stably low [Interval, $F_{(1, 8)}$=10.124, p=.013; Interval x Session, $F_{(11, 88)}$=7.392, p<.001]. Port entries during the NS, and PreNS intervals in the neutral context all remained stably low throughout conditioning relative to CS port entries in the alcohol context [Context x Interval, $F_{(1, 8)}$=9.340, p=.015]. Importantly, the increase in CS relative to PreCS port entries in the alcohol context, while port entries during all intervals in the neutral context remained low and stable, suggests that rats specifically acquired responding to the CS in the alcohol context [Interval x Context x Session, $F_{(11, 88)}$=7.293, p<.001; **Fig 3d** (left)].

The total number of port entries made during each training session was analyzed in a RM ANOVA including the factors Session (1, 2, 3, …12) and Context (Alcohol, Neutral). In the alcohol context, the total number of port entries were elevated relative to the neutral context

[Context, $F_{(1, 8)}$=23.119, p=.001]. Total port entries remained relatively stable in both the neutral and alcohol contexts across training sessions [Session, $F_{(11, 88)}$=1.051, p=.410; Context x Session, $F_{(11, 88)}$=1.285, p=.246; **Fig 3d** (right)].

After training, CS responding was tested by presenting the CS without alcohol in the alcohol and neutral context, after a microinfusion of vehicle or CNO (0.3 µl, 3 mM) according to a within-subjects design (**Fig 3e**). Test data were analyzed with an Interval (PreCS, CS) by Treatment (Vehicle, CNO) by Context (Alcohol, Neutral) RM ANOVA and NonCS port entries were analyzed in a separate RM ANOVA including the factors Treatment (Vehicle, CNO) and Context (Alcohol, Neutral). At test, CS port entries were elevated over PreCS port entries [Interval, $F_{(1, 8)}$=43.624, p<.001], a difference that was larger in the alcohol than the neutral context [Context, $F_{(1, 8)}$=9.183, p=.016; Context x Interval, $F_{(1, 8)}$=8.377, p=.02; **Fig 3f** (left)]. CNO microinfusion into the NAc shell did not affect CS port entries in the alcohol context or the neutral context [Treatment, $F_{(1, 8)}$=.028, p=.872; Treatment x Interval, $F_{(1, 8)}$=.014, p=.910; Context x Treatment, $F_{(1, 8)}$=.449, p=.552; Context x Treatment x Interval, $F_{(1, 8)}$=.183, p=.680; **Fig 3f** (left)]. NonCS port entries were also unaffected by CNO or context [Treatment, $F_{(1, 8)}$=3.326, p=.106; Context, $F_{(1, 8)}$=.067, p=.802; Context x Treatment, $F_{(1, 8)}$=1.384, p=.273; **Fig 3f** (right)].

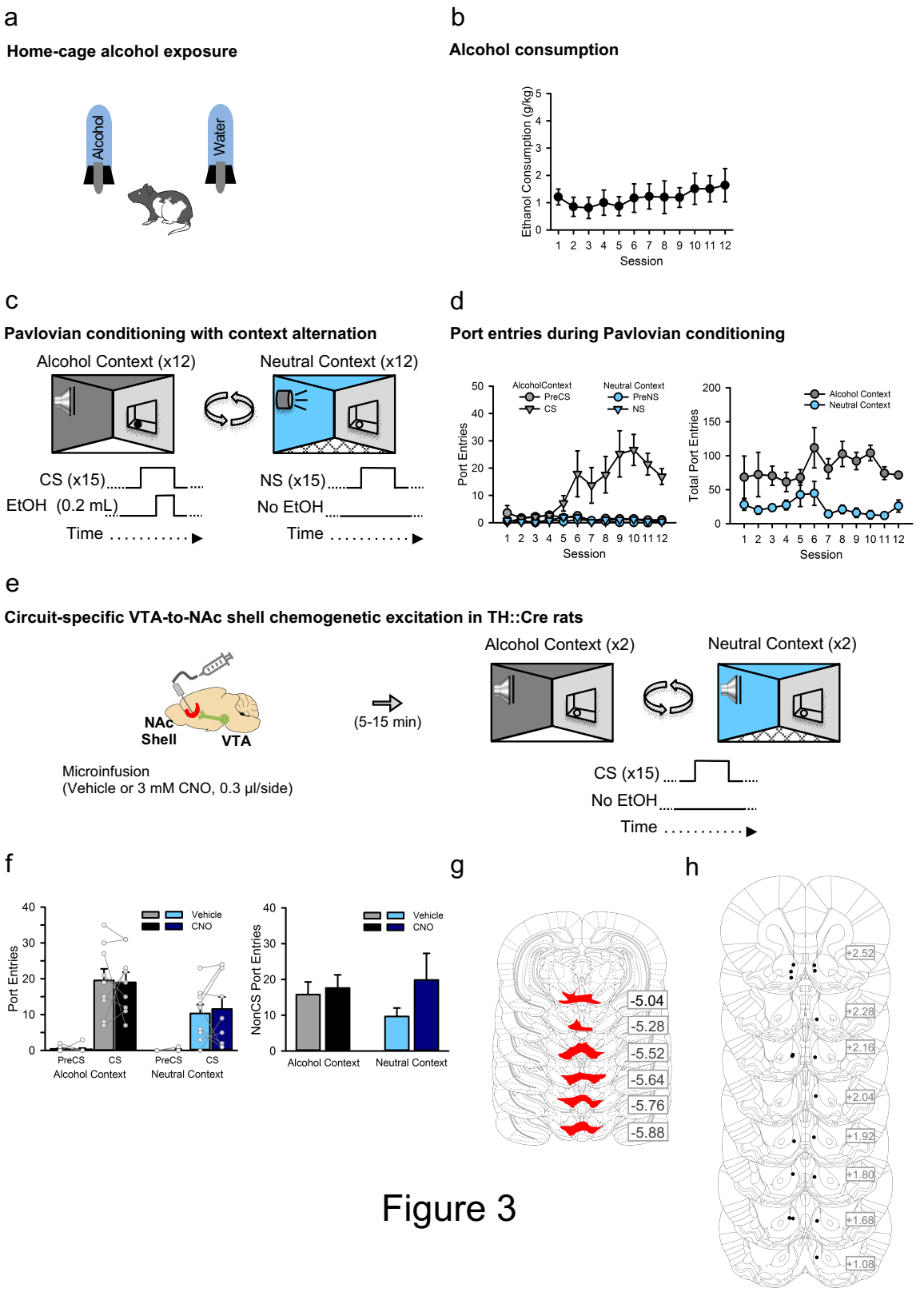

a
Home-cage alcohol exposure
Alcohol
Water
b
Alcohol consumption
Ethanol Consumption (g/kg)
Session
c
Pavlovian conditioning with context alternation
Alcohol Context (x12)
Neutral Context (x12)
CS (x15)
EtOH (0.2 mL)
Time
NS (x15)
No EtOH
Time
d
Port entries during Pavlovian conditioning
AlcoholContext
PreCS
CS
Neutral Context
PreNS
NS
Port Entries
Session
Alcohol Context
Neutral Context
Total Port Entries
Session
e
Circuit-specific VTA-to-NAc shell chemogenetic excitation in TH::Cre rats
NAc Shell
VTA
Microinfusion
(Vehicle or 3 mM CNO, 0.3 µl/side)
(5-15 min)
Alcohol Context (x2)
Neutral Context (x2)
CS (x15)
No EtOH
Time
f
Port Entries
Vehicle
CNO
PreCS
CS
PreCS
CS
Alcohol Context
Neutral Context
NonCS Port Entries
Vehicle
CNO
Alcohol Context
Neutral Context
g
-5.04
-5.28
-5.52
-5.64
-5.76
-5.88
h
+2.52
+2.28
+2.16
+2.04
+1.92
+1.80
+1.68
+1.08

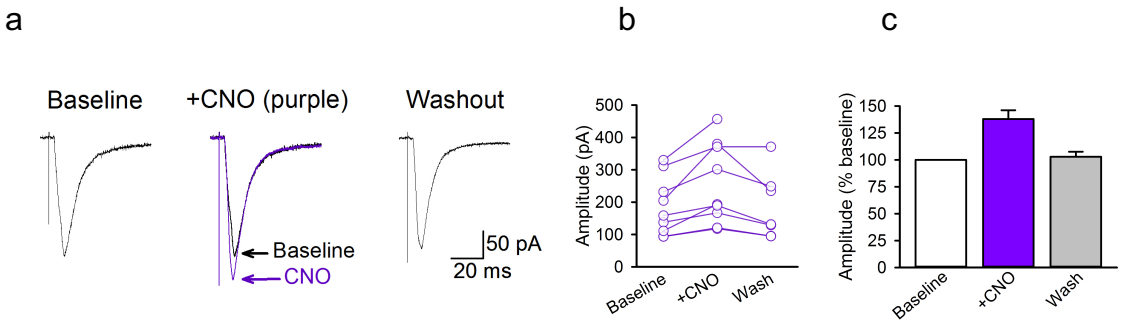
Figure 3

Chapter 3 Figure 3: Experiment 3. Chemogenetic excitation of a dopaminergic VTA-to-NAc shell circuit. **a** After recovering from surgery wherein TH::Cre rats (n=9) received microinfusion of a viral construct encoding the excitatory designer receptor (AAV8-hSyn-DIO-hM3Dq-mCherry) in the ventral tegmental area (VTA) and cannulae targeting the nucleus accumbens (NAc) shell, rats received 24 h access to a 15% ethanol (alcohol) solution in their home-cage every other day for a total of 12 sessions. Water and standard rat chow were continuously available. **b** Alcohol consumption shown as grams of ethanol over kilograms of body weight across 12 home-cage alcohol exposure sessions. **c** Then, rats received 12 Pavlovian conditioning sessions every other day in a distinct alcohol context wherein a discrete, 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (12 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. **d** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral context across sessions of Pavlovian conditioning with context alternation (right). **e** After Pavlovian conditioning with context alternation, rats received the first of 4 tests (2 in each context) for CS responding in the alcohol and neutral contexts which were conducted on separate days and intervened by retraining sessions. Before tests, rats received intracranial microinfusion of vehicle or CNO (0.3 µl, 3 mM, 0.3 µl/min). **f** PreCS and CS port entries (left) and NonCS port entries (right) during tests in the alcohol and neutral context. **g** Maximal mCherry expression in the VTA and **h** injector placements in the NAc shell for each rat is shown in schematics from the atlas of Paxinos & Watson (2008). Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 3

Experiment 4. Functional validation of circuit-specific chemogenetic excitation

To validate the ability of CNO to excite terminals of VTA dopamine neurons expressing excitatory designer receptors (hM3Dq) in the NAc, *in vitro* intracellular excitatory postsynaptic potentials in medium spiny neurons (MSNs) were recorded during bath-application of CNO. A 10-min period of CNO application significantly increased EPSCs to 137.94 ± 18.8% of baseline responses, from 186.1 ± 41.6 pA to 255.5 ± 41.6 pA [n=9, $F_{(2,14)}$=17.84, p<.001*; Newman-Keuls* p<.001; **Fig 4a-c**]. Responses returned to baseline values within 20 minutes of washout (102.87 ± 4.7 % of baseline values; **Fig 4 a-c**). Importantly, this finding confirmed that excitatory designer receptors expressed on VTA dopamine terminals in the NAc could, when activated by CNO, increase the activity of postsynaptic MSNs.



Chapter 3 Figure 4: Experiment 4. Functional validation of circuit-specific chemogenetic excitation. **a** Example traces show averaged excitatory post synaptic currents (EPSCs) for a NAc core medium spiny neuron before (baseline), during (+CNO), and after (washout) 10 min application of 1 μM CNO to striatal slices containing hM3Dq-expressing terminals from VTA dopamine neurons. **b** Peak amplitudes of EPSCs recorded from individual medium spiny neurons (MSNs) innervated by hM3Dq-expressing dopaminergic terminals. **c** Normalized mean EPSC amplitudes for the same group of MSNs. Averaged data are mean ± s.e.m.

Figure 4

Experiment 5. Effect of excitation of the NAc shell with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid on CS responding

In the absence of an effect of chemogenetic activation of the dopaminergic VTA-to-NAc shell projection on CS responding, the hypothesis that excitation produced by microinfusing a glutamate-like agonist in the NAc shell would impact CS responding was tested. Briefly, a group of naïve wild-type rats (n=13) were implanted with cannulae targeting the NAc shell, which were later used to deliver *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid* (AMPA) before tests for CS responding.

After recovering from surgery, rats received twelve sessions of exposure to alcohol in the home-cage. The dependent measure for home-cage alcohol exposure was grams of ethanol consumed as a function of rat weight in kilograms (g/kg). Alcohol consumption was analyzed using a RM ANOVA including the factor Session (1, 2, 3…12). Alcohol consumption was relatively stable across home-cage sessions [Session, $F_{(11, 110)}$=1.088, p=.379; **Fig 5b**].

Then, rats underwent Pavlovian conditioning with context alternation (**Fig 5c**). Port entries that occurred during the PreCS and CS intervals in the alcohol context, and the corresponding intervals in the neutral context were analyzed using a Session (1, 2, 3, …12) by Context (Alcohol, Neutral) by Interval (PreCS/PreNS or CS/NS) RM ANOVA. Port entries in the alcohol context increased across sessions relative to the neutral context [Session, $F_{(11, 110)}$=11.422, p<.001; Context, $F_{(1, 10)}$=51,229, p<.001; Context x Session, $F_{(11, 110)}$=15.883, p<.001]. The elevation in port entries in the alcohol context was driven by CS port entries which increased into a plateau, whereas port entries during the PreCS in the alcohol context remained stably low [Interval, $F_{(1, 10)}$=50.061, p<.001; Interval x Session, $F_{(11, 110)}$=14.704, p<.001]. Port entries during the NS, and PreNS intervals in the neutral context all remained stably low throughout conditioning relative to CS port entries in the alcohol context [Context x Interval, $F_{(1, 10)}$=50.347, p<.001]. Importantly, the increase in CS relative to PreCS port entries in the alcohol context, while port entries during all intervals in the neutral context remained low and stable, suggests that rats specifically acquired responding to the CS in the alcohol context [Interval x Context x Session, $F_{(11, 110)}$=15.319, p<.001; **Fig 5d** (left)].

The total number of port entries made during each training session was analyzed in a RM ANOVA including the factors Session (1, 2, 3, …12) and Context (Alcohol, Neutral). In the alcohol context, the total number of port entries were elevated relative to the neutral context

[Context, $F_{(1, 10)}$=75.102, p<.001]. Total port entries decreased to low levels across training sessions in the neutral context relative to the alcohol context [Session, $F_{(11, 110)}$=3.807, p<.001; Context x Session, $F_{(11, 110)}$=7.632, p<.001; **Fig 5d** (right)].

After training, CS responding was tested by presenting the CS without alcohol in the alcohol and neutral context, after a microinfusion of vehicle or AMPA (0.3 µl, 0.1 mM) according to a within-subjects design (**Fig 5e**). Test data were analyzed with an Interval (PreCS, CS) by Treatment (Vehicle, AMPA) by Context (Alcohol, Neutral) RM ANOVA and NonCS port entries were analyzed in a separate RM ANOVA including the factors Treatment (Vehicle, AMPA) and Context (Alcohol, Neutral). At test, CS port entries were elevated over PreCS port entries [Interval, $F_{(1, 10)}$=30.147, p<.001], a difference that was larger in the alcohol than the neutral context [Context, $F_{(1, 10)}$=15.360, p=.003; Context x Interval, $F_{(1, 10)}$=20.332, p=.001; **Fig 5f** (left)]. AMPA microinfusion into the NAc shell reduced CS port entries in the alcohol context but not the neutral context [Treatment, $F_{(1, 10)}$=7.348, p=.022; Treatment x Interval, $F_{(1, 10)}$=7.611, p=.02; Context x Treatment, $F_{(1, 10)}$=7.192, p=.023; Context x Treatment x Interval, $F_{(1, 10)}$=5.158, p=.046; **Fig 5f** (left)]. Post-hoc Bonferroni-corrected t-tests confirmed that CS port entries were significantly lower in the alcohol context after AMPA microinfusion [$t_{(10)}$=2.961, p=.014] but not in the neutral context [$t_{(10)}$=.433, p=.674].

NonCS port entries were increased in the neutral, but not alcohol, context by AMPA and higher in the alcohol than in the neutral context [Treatment, $F_{(1, 10)}$=.041, p=.843; Context, $F_{(1, 10)}$=5.431, p=.042; Context x Treatment, $F_{(1, 10)}$=9.637, p=.011; **Fig 5f** (right)]. Post-hoc Bonferroni-corrected t-tests confirmed that NonCS port entries were significantly elevated in the neutral context after AMPA microinfusion [$t_{(10)}$=-3.907, p=.003] but remained unchanged in the alcohol context [$t_{(10)}$=1.456, p=.167].

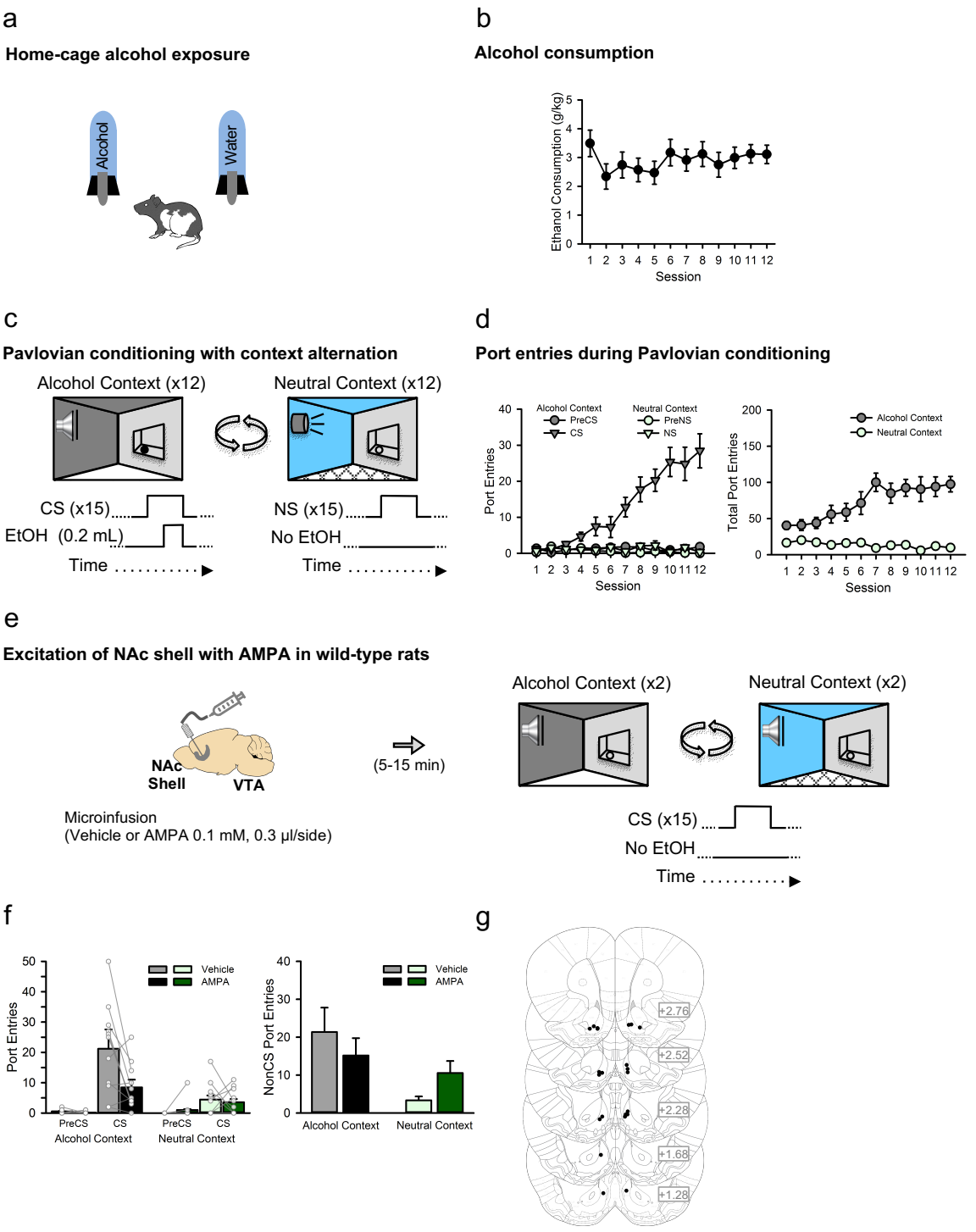

a
Home-cage alcohol exposure
Alcohol
Water
b
Alcohol consumption
Ethanol Consumption (g/kg)
Session
c
Pavlovian conditioning with context alternation
Alcohol Context (x12)
Neutral Context (x12)
CS (x15)
EtOH (0.2 mL)
Time
NS (x15)
No EtOH
Time
d
Port entries during Pavlovian conditioning
Alcohol Context
PreCS
CS
Neutral Context
PreNS
NS
Port Entries
Session
Alcohol Context
Neutral Context
Total Port Entries
Session
e
Excitation of NAc shell with AMPA in wild-type rats
NAc Shell
VTA
(5-15 min)
Microinfusion
(Vehicle or AMPA 0.1 mM, 0.3 µl/side)
Alcohol Context (x2)
Neutral Context (x2)
CS (x15)
No EtOH
Time
f
Vehicle
AMPA
Port Entries
PreCS
CS
PreCS
CS
Alcohol Context
Neutral Context
Vehicle
AMPA
NonCS Port Entries
Alcohol Context
Neutral Context
g
+2.76
+2.52
+2.28
+1.68
+1.28

Figure 5

Chapter 3 Figure 5: Experiment 5. Effect of excitation of the NAc shell with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid on CS responding in the neutral and alcohol context. **a** After recovering from surgery wherein wild-type rats (n=13) received cannulae targeting the nucleus accumbens (NAc) shell, rats received 24 h access to a 15% ethanol (alcohol) solution in their home-cage every other day for a total of 12 sessions. Water and standard rat chow were continuously available. **b** Alcohol consumption shown as grams of ethanol over kilograms of body weight across 12 home-cage alcohol exposure sessions. **c** Then, rats received 12 Pavlovian conditioning sessions every other day in a distinct alcohol context wherein a discrete, 10 s auditory conditioned stimulus (CS; 15 trials per session; variable inter trial interval = 260 s) was paired with alcohol (0.2 ml per CS; 3 ml per session) delivery into a fluid port for oral consumption. On alternating days, rats were exposed to a different, neutral context (12 sessions) where a distinct, 10 s neutral auditory stimulus (NS; 15 trials per session) was presented without alcohol. **d** Port entries during the PreCS and CS periods in the alcohol context and during the NS and PreNS periods in the neutral context across sessions of Pavlovian conditioning with context alternation (left). Total port entries in the alcohol and neutral context across sessions of Pavlovian conditioning with context alternation (right). **e** After Pavlovian conditioning with context alternation, rats received the first of 4 tests (2 in each context) for CS responding in the alcohol and neutral contexts which were conducted on separate days and intervened by retraining sessions. Before tests, rats received intracranial microinfusion of vehicle or AMPA (0.3 µl, 0.1 mM, 0.3 µl/min). **f** PreCS and CS port entries (left) and NonCS port entries (right) during tests in the alcohol and neutral context. **g** Injector placements in the NAc shell for each rat is shown in schematics from the atlas of Paxinos & Watson (2008). Averaged data are mean ± s.e.m. Data from individual rats are shown as grey circles overlaid on bar graphs.

Figure 5

Discussion

Systemically administered dopamine antagonists reduce responding in animal models of alcohol relapse[53,71,72,85], suggesting that the dopamine system is necessary for responding to alcohol cues, however, it is unknown whether dopaminergic activity is sufficient to elevate responding to alcohol cues. Chapter 3 endeavoured to examine the hypotheses that activating VTA dopamine neurons, their projections to the NAc shell, or increasing glutamatergic activity in the NAc shell might serve to elevate responding to a discrete alcohol CS. In experiment 1, chemogenetic activation of VTA dopamine neurons failed to affect responding to a discrete alcohol CS in a neutral context, despite reducing feeding behaviour (Exp. 2) in the same rats. Using a circuit-specific chemogenetic approach to activate the dopaminergic VTA-to-NAc shell projection also failed to impact responding to a discrete alcohol CS (Exp. 3). Importantly, the circuit-specific approach was validated using electrophysiology (Exp. 4). In the absence of an effect of dopaminergic activity on CS responding, the capacity for increased glutamatergic activity in the NAc shell to affect CS responding was examined using AMPA. Interestingly, AMPA microinfusion into the NAc shell selectively reduced CS responding in an alcohol context, but not in the neutral context, further highlighting a role for the NAc shell in the influence of context over CS responding (Exp. 5). In sum, although dopaminergic activity is necessary for responding to a discrete alcohol CS, it may not be sufficient to elevate responding to an alcohol CS, and glutamatergic activity in the NAc shell is critical for responding to a discrete alcohol CS in an alcohol context.

In experiment 1, chemogenetic activation of VTA dopamine neurons did not affect responding to a discrete alcohol CS, suggesting that VTA dopamine activity was not sufficient to elevate responding to a discrete alcohol CS. To rule out the possibility that this null effect could be explained by technical failure of the chemogenetic approach, the capacity for VTA dopaminergic activity to reduce feeding was examined. It had been demonstrated previously, that chemogenetic activation of VTA dopamine neurons reduced food consumption during the first two hours of food access under a restricted feeding schedule[157]. This effect was replicated, in that CNO acting on excitatory designer receptors expressed on VTA dopamine neurons reduced food consumption during the first two hours of food access but had no effect in a control group of rats without designer receptors (Exp. 2). As an extension to this replication, food consumption during the entire feeding window and water consumption were also analyzed. Interestingly, the initial suppression in feeding behaviour persisted throughout the entire feeding window (16 h), whereas water consumption remained unaffected. Together, the results from

experiments 2 and 3 suggested that chemogenetic activation of VTA dopamine neurons was capable of producing a long-lasting effect on consummatory behaviour, but was insufficient to influence responding to a discrete alcohol CS.

Although the feeding study conducted in experiment 2 was secondary to the main investigation of this thesis, it revealed some important technical insights regarding the use of clozapine as a designer ligand. The capacity for clozapine to act on designer receptors was documented in the original report of this receptor[130], however the potential use of clozapine as a designer ligand gained popularity when it was discovered that CNO could be reverse metabolized (~1-2%) to clozapine in rodents[135,137,139]. As a proof of concept that reverse metabolism of CNO to clozapine could have behavioural consequences through actions on designer receptors, it was demonstrated that clozapine suppressed locomotor activity in rats expressing the inhibitory designer receptor throughout the striatum and basal forebrain[135]. Although, relatively low levels of clozapine in blood plasma and cerebrospinal fluid are achieved through reverse metabolism[139], clozapine has a high affinity for designer receptors and thus can bind to and activate designer receptors even at low concentrations[130]. It has also been argued that clozapine's high affinity for designer receptors allows it to be used as a designer ligand at concentrations that do not produce appreciable off-target binding and unintended pharmacological actions[137]. In the current thesis, CNO (10 mg/kg) and a 100-fold lower dose of clozapine (0.1 mg/kg) reduced feeding behaviour comparably through actions on designer receptors expressed in the VTA, as there were no effects on feeding or drinking behaviour in rats expressing a control fluorescent protein. Thus, clozapine is an economical and efficacious designer ligand for chemogenetic studies.

The lack of an effect of increased VTA dopamine activity on responding to an alcohol CS may indicate that the role of dopaminergic activity in driving CS responding is projection specific. Certainly, the finding that VTA-to-NAc shell activity was necessary for responding to a discrete alcohol CS in an alcohol context (Ch 2 Exp. 5) implicates this projection in the influence of context over CS responding. Further, the capacity for dopamine microinfused directly into the NAc (core and shell not separated) to reinstate extinguished cocaine-seeking[187] suggests that a similar chemogenetic manipulation may elevate Pavlovian responding for alcohol. However, in experiment 3 chemogenetic activation of the dopaminergic VTA-to-NAc shell projection failed to affect responding to a discrete alcohol CS in either the alcohol or neutral context. One explanation for the lack of an effect of chemogenetically activating VTA dopamine neurons (Exp.

1) or their projection to the NAc shell (Exp. 3) is that responding had hit a ceiling. However, this explanation is unlikely because rats made ~30-40 port entries in the alcohol context in previous experiments, and ~20 in the neutral context in experiment 1. Further, CS responding was tested in the alcohol and neutral contexts in experiment 3, confirming that while CS responding in the neutral context remained low and was unaffected by VTA-to-NAc shell activation, this group of rats was capable of elevating their responding in the alcohol context. In summary, neither chemogenetic activation of VTA dopamine neurons or their projections to the NAc shell impacted responding to a discrete alcohol CS.

Although chemogenetic activation of the VTA-to-NAc shell projection was ineffective at elevating responding to a discrete alcohol CS, this circuit-specific chemogenetic approach was validated using electrophysiology, ruling out a technical failure of the approach. In experiment 4, electrically-evoked EPSCs were recorded from NAc core MSNs that were innervated by dopaminergic terminals expressing the excitatory designer receptor. CNO acting on these designer receptors increased the amplitude of EPSCs in NAc core MSNs. An important caveat of this validation experiment is that EPSCs were recorded from the NAc core whereas the *in vivo* manipulations took place in the NAc shell. However, there is not an obvious reason to assume that electrically-evoked EPSCs differ between the core and shell subregions, and it has been shown previously that optogenetic stimulation of VTA dopamine terminals in the NAc shell independently evoke EPSCs in MSNs[176,179]. Further, increases in EPSC amplitude following chemogenetic activation of VTA dopamine terminals in the NAc core is consistent with work demonstrating that this activation evokes dopamine release in the NAc[134]. Specifically, dopamine release in the NAc can act synergistically at D1 and D2 receptors on MSNs to enhance MSN activity[170,171]. Furthermore, increased glutamate levels in the NAc, achieved either through corelease from VTA dopamine neurons[176–178] or dopaminergic modulation of corticostriatal glutamate release[76,170,172–175], can increase MSN activity. Importantly, experiment 4 provides evidence that the excitatory circuit-specific chemogenetic approach was capable of increasing postsynaptic MSN activity.

Glutamatergic inputs to the NAc shell are necessary for the renewal of alcohol-seeking behaviour[70], and in the absence of effects of dopaminergic activity on responding to a discrete alcohol CS, the role of glutamatergic activity in the NAc was investigated. Interestingly, microinfusion of AMPA and dopamine into the NAc shell reinstate extinguished cocaine-seeking behaviour and this reinstatement is blocked by an AMPA antagonist[185]. The capacity for an

AMPA antagonist to block dopamine-induced reinstatement of cocaine-seeking highlights an interplay between glutamate and dopamine in the NAc shell that is relevant for drug-seeking behaviour[75–78,191]. Thus, it was reasoned that increasing glutamatergic activity in the NAc shell may be sufficient to elevate responding to a discrete alcohol CS. This possibility was tested by microinfusing AMPA into the NAc shell before tests for CS responding in the alcohol and neutral contexts. Interestingly, AMPA microinfusion in the NAc shell selectively reduced CS responding in the alcohol context and not in the neutral context (Exp. 5). While this reduction was unexpected, the selective effect of AMPA in the NAc shell on CS responding in the alcohol context, underscores the importance of the NAc shell for responding to discrete alcohol cues in alcohol contexts.

AMPA microinfusion into the NAc shell left CS responding unaffected in the neutral context, but elevated NonCS port entries in this context. NonCS port entries may reflect unconditioned locomotor or exploratory behaviour because responding during this interval is unreinforced, especially in well trained rats that are highly sensitive to the timing of US delivery[192]. The finding that AMPA elevated NonCS port entries in the neutral context is difficult to reconcile with the report that NAc shell inactivation (muscimol/baclofen) increased NonCS port entries in the neutral context[73]. However, both of these findings point to a role for the NAc shell in permitting behaviour. There is a population of NAc MSNs, including those in the shell, that become inhibited immediately before and during appetitive and consummatory behaviour[193,194]. It is thought that this population of neurons acts to suppress behaviour at inappropriate times and becomes inhibited to permit appetitive and consummatory behaviour. In the current procedure a population of NAc shell MSNs may become inhibited during CS presentations to permit CS port entries. AMPA may interfere with the transition of these MSNs between active and inhibited states and thus increase responding outside the CS interval, perhaps specifically prolonging responding after CS presentations and into the NonCS interval.

All attempts to elevate responding to a discrete alcohol CS by activating dopaminergic and glutamatergic substrates, either failed to affect or decreased CS responding. Despite these unanticipated results, some insights about the neural substrates that underpin responding to a discrete alcohol CS in different context were generated. For example, NAc levels of glutamate are elevated by the presentation of alcohol cues and during the reinstatement of alcohol-seeking behaviour[195], yet AMPA microinfusion in the NAc shell in experiment 5 decreased responding to a discrete alcohol CS. Interestingly, this effect was context specific, and suggests

that the capacity for an alcohol context to elevate responding to a discrete alcohol CS depends on glutamatergic activity in the shell.

The use of AMPA to mimic glutamatergic activity in the NAc shell in experiment 5 also addresses the hypothesis that elevated dopaminergic activity in the NAc shell would augment responding to an alcohol CS. Microinfusion of AMPA into the NAc elevates extracellular dopamine levels as detected by voltammetry[78]. Thus, the hypothesis that elevating NAc shell dopamine levels by activating VTA inputs could drive responding to an alcohol CS, which was unsupported by chemogenetic VTA-to-NAc shell activation (Exp. 3), remains unsupported by the result that AMPA in the NAc shell reduced or did not affect CS responding in the alcohol and neutral contexts, respectively (Exp. 5). However, there are limitations on the temporality and specificity with which chemogenetic and pharmacological approaches serve to manipulate dopaminergic and glutamatergic activity in the NAc shell, which might explain the failure to elevate CS responding in chapter 3. If there is a role for increased glutamatergic and dopaminergic signalling in the NAc shell in driving responding to alcohol cues, then these substrates are probably involved in a temporally- and input-specific manner. An optogenetic approach could be used to stimulate glutamatergic or dopaminergic inputs to the NAc shell during CS presentations, or other relevant behavioural epochs, which might serve to elevate responding to a discrete alcohol CS.

Another hypothesis regarding the sufficiency of glutamatergic and dopaminergic input to the NAc shell to elevate responding to a discrete alcohol CS, is that convergent activation of these projections is required to elevate CS responding. As a first step in pursuing this hypothesis a subgroup of rats from experiment 3 received an infusion of a viral construct encoding only a fluorescent protein in the vSub. This was done to ensure that expressing a viral construct in the vSub, in addition to excitatory designer receptors expressed on VTA dopamine neurons, did not impact behaviour. The vSub was targeted because inputs from the vSub to the NAc shell are necessary for the renewal of alcohol-seeking behaviour[70]. It was also demonstrated in this thesis that VTA-to-NAc shell dopaminergic activity was necessary for responding to a discrete alcohol CS in an alcohol context (Ch 2 Exp. 5). Thus, it seemed that glutamatergic input from the vSub and dopaminergic input from the VTA might, when convergently active, serve to elevate responding to a discrete alcohol CS. This convergent activity hypothesis is uniquely testable using a chemogenetic approach. Excitatory designer receptors could be expressed on VTA dopamine neurons and vSub neurons in TH::Cre rats,

resulting in the trafficking of these receptors to terminals the NAc shell where a microinfusion of CNO would activate both inputs simultaneously. An important compliment to this hypothetical study would be to express an inhibitory designer receptor on one of the projections and test whether inactivation of one project nulled the behavioural effect achieved through convergent activation. The use of chemogenetics to simultaneously influence the activity of different brain areas has been done before[196], but the capacity to simultaneously modulate the activity of two separate projections remains untapped.

The main aim of chapter 3 was to examine to capacity for activation of dopaminergic or glutamatergic substrates to elevate responding to a discrete alcohol CS. Ultimately, this endeavor was unsuccessful as chemogenetic excitation of VTA dopamine neurons (Exp. 1), their projections to the NAc shell (Exp. 3), and increased glutamatergic activity in the NAc shell (Exp. 5) all failed to elevate responding to a discrete alcohol CS. However, important insights were generated about the role of VTA dopamine activity in supressing feeding behaviour, which was shown to be a longer lasting effect than previously thought[157]. Further, the capacity for clozapine to act as an efficacious designer ligand was demonstrated (Exp. 2). Also, an important validation of the an excitatory chemogenetic approach showed that CNO acting on excitatory designer receptors on VTA dopamine terminals could influence the activity of postsynaptic MSNs (Exp. 4). Lastly, AMPA was used to mimic glutamatergic activity in the NAc shell and revealed that this activity reduced responding to a discrete alcohol CS in an alcohol context but left CS responding in the neutral context unaffected (Exp. 5). Thus, glutamatergic activity in the NAc shell is implicated in responding to a discrete alcohol CS in a context-specific manner. The question of which neural substrates are sufficient to elevate responding to an alcohol CS, unfortunately remains unknown, however the experiments presented in chapter 3 offer a basis for future studies in pursuit of an answer.

General Discussion

There is a rich history of preclinical research describing a role for the dopamine system in substance use disorders[197–199] that continues to permeate contemporary thinking[200]. In part, this view is supported by the finding that most substances of abuse, including alcohol, elevated dopamine levels in the NAc[198,199] and that dopamine antagonists attenuated drug self-administration in rats[197,201–203]. Once more sophisticated animal models were developed to study relapse, like cue-induced reinstatement[24,27] and context renewal[44,46], researchers sought to determine whether dopamine antagonists also reduced this behaviour. Indeed, dopamine antagonists were effective at reducing responding in the cue-induced reinstatement and context renewal paradigms across a variety of drug reinforcers[25,48]. Preclinical investigations of relapse became more refined as researchers focused on the necessity of specific subcomponents of the dopamine system in responding that was influenced by different types of environmental stimuli, like discrete cues and contexts, often inventing new animal models to do so[55,56,108,168,204,205]. The experiments presented in the current thesis are an extension of this line of research, aiming to characterize the influence of discrete cues and contexts over responding for alcohol (Ch 1), uncover the roles of dopaminergic projections from the VTA to the NAc core and shell in this behaviour (Ch 2), and determine whether activation of a dopaminergic substrate is sufficient to elevate responding to a discrete alcohol CS (Ch 3).

The capacity for context to influence responding to a discrete alcohol CS

A critical step in understanding the capacity for environmental cues to engender alcohol-seeking is to describe the extent to which the isolated and combined presentation of discrete cues and contexts affect behaviour. Chapter 1 used a Pavlovian conditioning procedure to demonstrate that responding to a discrete alcohol CS was elevated in an alcohol-associated context relative to a neutral context that was equated in terms of familiarity and acoustic salience (Ch 1 Exp. 1a). Further, this elevation of CS responding in the alcohol context was shown to be long-lasting as it persisted across multiple repeated test sessions (Ch 1 Exp. 1b) and re-emerged in a reinstatement test (Ch 1 Exp. 1c). A detailed analysis of CS responding at test in the alcohol and neutral contexts (Ch 1 Exp. 1), and an additional experiment (Ch 1 Exp. 2) designed to examine the timing of responses to a discrete CS, described the minute features of CS responding that were influenced by context. Collectively, chapter 1 provided a detailed behavioural account of the robustness, persistence, and temporal sensitivity with which an alcohol context affects responding to a discrete alcohol CS.

The robustness of the principle behavioural effect (Ch 1 Exp. 1), that port entries during a discrete alcohol CS are elevated in the alcohol context relative to the neutral context, was replicated across multiple experiments in this thesis. In chapter 1, two separate groups of wild-type rats demonstrated a similar effect of context at test, despite only one group receiving a NS in the neutral context during training (Ch 1 Exp. 1a). This first experiment, which addressed a context inequity in acoustic salience in previous studies[57,73], was a within-experiment replication of the capacity for context to elevate CS responding. In separate experiments, TH::Cre rats that expressed designer receptors on VTA dopamine neurons and were implanted with cannulae targeting the NAc core (Ch 2 Exp. 4) or shell (Ch 2 Exp. 5, Ch 3 Exp. 3) demonstrated a similar effect of context on CS responding. Lastly, a group of wild-type rats with cannulae targeting the NAc shell also replicated the effect of context on CS responding (Ch 3 Exp. 5). Together, the principal behavioural effect in this thesis, that responses to a discrete alcohol CS were elevated in an alcohol context relative to a neutral context, was replicated within and between experiments in transgenic and wild-type rats under a variety of surgical preparations.

The capacity for context to elevate responding to a discrete CS was persistent. After an initial test for CS responding in the alcohol and neutral contexts (Ch 1 Exp. 1a), CS port entries were maintained at a higher level for longer in the alcohol context than in the neutral context across a series of repeated alcohol-free test sessions (Ch 1 Exp. 1b). Specifically, CS port entries displayed peaks and troughs that corresponded to the alternation of repeated test sessions from the alcohol, to the neutral context, respectively. At the end of the repeated test sessions, CS responding had waned to low levels in both contexts suggesting that the capacity for the discrete CS to elicit responses, and for context to influence these responses, had extinguished. After extinction, an oral alcohol prime, which had not undergone extinction, was capable of reinstating CS responding to a higher level in the alcohol context than in the neutral context (Ch 1 Exp. 1c). Thus, CS responding was resistant to extinction and reinstated to a higher level in the alcohol context, demonstrating the persistence of the capacity for an alcohol context to elevate CS responding.

The resistance of CS responding to extinction and elevated reinstatement in the alcohol context has implications for understanding the role of learning processes in alcohol use disorder. In cue-exposure therapy people are repeatedly exposed to alcohol cues to extinguish reactivity (e.g. craving, desire to drink) to these cues with the goal of preventing a relapse episode when people experience these cues in the future[19,20,206–208]. The result in the current

thesis that an oral alcohol prime reinstated context-dependent CS responding after extinction supports a well-established hypothesis that extinction imparts new learning, instead of erasing old learning[140]. The original learning about the associations between the discrete CS and alcohol context with alcohol was not lost during extinction, indicating that interpreting extinguished cue-reactivity as a measure of relapse potential is an overestimation. Further, the learning that occurs during cue-exposure sessions is likely context-dependent[141]. While cue reactivity may appear low in a clinic or lab setting, it is not clear whether this low reactivity will remain in an alcohol-associated context. Moreover, the capacity for an oral alcohol prime to reinstate CS responding, and the influence of context over this responding, suggests that extinguishing reactivity to one cue, even across contexts, does not necessarily prevent a non-extinguished cue from triggering relapse. It has been demonstrated previously that realistic cue-exposure sessions are most effective at developing a resistance to cue-induced alcohol consumption in people[22]. Further consideration of the setting of cue-exposure sessions and the multiplicity of alcohol cues that have the capacity to trigger relapse may improve the efficacy of cue-exposure therapy.

A detailed analysis of responding at test in the alcohol and neutral contexts provides insights into the learning processes that govern the influence of context over CS responding. The principal finding that CS port entries were elevated in the alcohol context relative to the neutral context when the CS was presented without alcohol did not extend to NonCS port entries which were similar in both contexts at test (Ch 1 Exp. 1a). CS port entries clearly reflect conditioned behaviour because they demonstrate an acquisition curve during training and persist in the absence of the US (alcohol) at test. However, NonCS port entries could be indicative of general port-directed behaviour or unconditioned locomotor and exploratory behaviour, or direct context conditioning. NonCS port entries tend not to differ between the alcohol and neutral contexts during tests (Ch 1 Fig 2b, Ch 2 Fig 4f, Ch 3 Fig 3f, but see Ch 2 Fig 5f and Ch 3 Fig 5f). The lack of an effect of context on NonCS port entries in the current procedure, may indicate that context does not directly elicit a conditioned response, but rather influences the association between the discrete CS and US (alcohol). More specifically, the analysis of CS port entries across trials at test, suggests that context stably influences the magnitude of responding across trials (Ch 1 Fig 2c), without affecting other properties of the response form, like latency and duration (Ch 1 Fig 2d-e). The capacity for the alcohol context to elevate CS port entries without affecting general port-directed behaviour, or other features of the response form, are clues as to the learning processes that govern the influence of context over

responses elicited by a discrete alcohol CS and are consistent with a learning process called occasion setting.

Occasion setting is a theoretical account of the capacity for a stimulus to exert control over responding to a CS through a mechanism that is separate from the direct association between that stimulus and the US[209]. Occasion setters were established as separate from simple CSs using feature-target conditioning procedures wherein a stimulus termed the feature determined whether a subsequently presented CS (called a target in this literature) would be reinforced. In feature-positive conditioning a CS is reinforced only if it is preceded by the feature and is unreinforced when presented alone. Inversely, in feature-negative conditioning the CS is reinforced when it is presented alone and is unreinforced when preceded by the feature. Three interesting properties of features emerged that are unique and must be met to qualify a stimulus as an occasion setter.

Firstly, occasion setters control the magnitude, but not the form of a CS response[209–211]. For example, rearing and orienting are responses typically generated by auditory and visual stimuli, respectively. When the feature is an auditory stimulus and the CS is a visual stimulus, rats will orient to the CS, even though the feature indicates whether the CS will be reinforced[212,213]. Therefore, occasion setters influence the magnitude, but not the form, of a response elicited by a CS.

Secondly, occasion setters modulate CS-US associations independently of their direct association with the US. For example, in a feature-positive conditioning procedure extinguishing the direct excitatory association between the feature and the US by presenting the feature alone and unreinforced, does not impact the excitatory control that the feature exhibits over the CS response[212]. Further, in a feature-negative procedure, reinforcing presentation of the feature alone does not alter the capacity of the feature to inhibit CS responding[214,215]. Thus, the direct association between an occasion setter (feature) and the US is independent of the capacity for the occasion setter to modulate the CS-US association.

Thirdly, unlike simple CSs which summate to excite or inhibit responding when presented concominantly[6,216–218], the capacity for an occasion setter to modulate a CS-US association does not transfer to other CSs[209–211,219]. In either feature-negative or -positive

conditioning procedures the capacity for a feature to influence CS responding will not summate with the direct excitatory or inhibitory influence of a separately conditioned CS[209,213,215§].

In the present thesis, the capacity for context to elevate responses to a discrete alcohol CS resembles occasion setting. While it is difficult to interpret the nature of NonCS port entries, it is clear that they typically remain unaffected by context in the Pavlovian conditioning with context alternation procedure. If NonCS port entries are taken as a measure of direct context conditioning between the context and the US (alcohol), then it is clear that the alcohol context does not elicit a direct excitatory response because there is no difference in NonCS port entries across contexts. The lack of a direct excitatory response by the alcohol context suggests that the alcohol context does not elevate responses to the discrete alcohol CS through summation of excitatory responses elicited by the alcohol context and the discrete alcohol CS, but rather through modulation of the association between the discrete alcohol CS and the US (alcohol).

Another characteristic of the alcohol context that resembles occasion setting is that the latency and duration of CS port entries, which may be considered properties of the response form, do not differ between the neutral and alcohol context. That is, the alcohol context elevates CS responses without affecting properties of the response form. Importantly, the lack of an effect of context on the latency and duration of CS port entries cannot be attributed to the inability of context to affect the timing of responses elicited by a discrete alcohol CS. It was demonstrated in this thesis (Ch 1 Exp. 2) using a conditioning procedure wherein context determined the timing of US (alcohol) delivery during a CS that context has the capacity to control the timing of responses elicited by a discrete alcohol CS. The capacity for the alcohol context to elevate CS port entries without affecting NonCS port entries, the latency of CS port entries, or the duration of CS port entries resemble the characteristics of an occasion setter.

A critical property of occasion setters is that their capacity to influence responding to a discrete CS is unaltered when the direct association between the occasion setter and the US is extinguished[209,220], however it is not possible to adequately address this property with the experiments in the current thesis. After CS responding was tested in the alcohol and neutral contexts (Ch 1 Exp. 1a), CS responding was extinguished by presenting the CS without alcohol in both the alcohol and neutral contexts on alternating days. Unreinforced presentations of the alcohol CS in both the alcohol and neutral contexts likely extinguished the direct association

§ Results from both studies shown together in Holland (1992) p. 79.

between the discrete CS and alcohol, and the alcohol context and alcohol. Thus, it was not possible to adequately assess whether extinguishing the direct association between the alcohol context and alcohol left intact the capacity for the alcohol context to elevate CS responding, because the CS-US association was also extinguished. As such, it is difficult to determine whether the reinstatement of CS responding, which was elevated in the alcohol context relative to the neutral context, confirms that the alcohol context served as an occasion setter. However, the reinstatement result is not inconsistent with the hypothesis that the alcohol context is an occasion setter.

The finding that at times the alcohol context seems to elevate NonCS port entries (Ch 2 Fig 5f and Ch 3 Fig 5f), appears inconsistent with the suggestion that the alcohol context is an occasion setter, however this finding can be explained by variability in learning strategies adopted by individual rats. A unique property of Pavlovian conditioning with context alternation, is that rats are not required to learn an association between the alcohol context and alcohol to successfully obtain alcohol when it is delivered during the CS[38]. As such, rats may learn different things about the alcohol context: a direct excitatory association between the alcohol context and alcohol, that the discrete CS predicts alcohol and to ignore context, or that the alcohol context is an occasion setter. The variability in individual trajectories of responding to the CS across the alcohol and neutral contexts supports this idea, as some rats show large changes in CS responding across contexts, whereas other rats show only modest differences in CS responding or no change at all. Relatedly, the capacity for context to renew cocaine-seeking behaviour varies cross two subpopulations of rats[221] which suggests that rats vary in their propensity to use contextual cues to orchestrate responding for drug reinforcers. In the current procedure the distribution of rats across different learning strategies may particularly affect NonCS port entries. If a large proportion of rats learn a direct excitatory association between the alcohol context and alcohol, and if this learning manifests in NonCS responding, then the alcohol context may elevate NonCS port entries. The adequacy of the hypothesis that rats adopt different learning strategies during Pavlovian conditioning with context alternation can be examined by conducting hierarchical cluster analyses of CS and NonCS port entries in the alcohol and neutral context at test and to observe whether rats are naturally distributed into two or three clusters. In sum, the hypothesis that the alcohol context acts as an occasion setter is consistent with most of the behavioural findings in this thesis and it is an open question whether alternative learning strategies are adopted by subgroups of rats.

Historically, the criteria used to establish a stimulus as an occasion setter were described using brief, serial, feature (occasion setter) and target (CS) presentations, and it is difficult to translate these criteria to contexts which differ substantially in modality from discrete cues[209,211,220,222,223]. For example, in the current procedure it is difficult to conduct an extinction test of context, because context does not seem to elicit a direct excitatory response (NonCS), and further, context extinction can be difficult to disentangle from habituation[104,224]. Nevertheless, the behavioural findings from various context manipulations resemble occasion setting. Specifically, the alcohol context appears to elevate CS port entries by influencing the CS-US (alcohol) association without affecting NonCS port entries, this influence survives extinction, and the alcohol context does not influence response form. These findings do not conclusively establish the alcohol context as an occasion setter but are consistent with the hypothesis that the alcohol context acts as an occasion setter to influence responding to a discrete alcohol CS in the Pavlovian conditioning with context alternation procedure.

The role of the dopamine system in responding to a discrete alcohol CS

The involvement of the mesolimbic dopamine system in alcohol use disorder, is undeniable. The mesolimbic dopamine system is dysregulated in people with a history of alcohol misuse[83], activated by alcohol in rats and humans[80,82], and active during the presentation of discrete alcohol cues[126] and contexts[79] in rats. Further, systemically administered dopamine antagonists reduce responding for alcohol in animal models of relapse[53,85,106]. In the current thesis pharmacology and chemogenetic approaches were used to test the necessity of the dopamine system and VTA dopamine neurons, respectively, for responding to a discrete alcohol CS. Further, a chemogenetic approach was used to test the sufficiency of VTA dopamine activity to elevate responding to a discrete alcohol CS. Importantly, Pavlovian conditioning with context alternation allowed for responding to a discrete alcohol CS to be tested in a familiar neutral context, that had no association with alcohol. Together, these experiments reveal that VTA dopamine neurons play an important and selective role in responding to discrete alcohol cues.

An important step in targeting VTA dopamine neurons was to validate the use of cre-dependent chemogenetics in TH::Cre rats. Frist, the selectivity for a cre-dependent (DIO) designer receptor construct to target TH positive neurons in the VTA was examined. Designer receptor expression encompassed the anterior-posterior and medio-lateral expanse of the VTA, within which cells are organized along a medio-lateral gradient depending on their projection

target region. Specifically, cell bodies located near the midline project predominantly to medial areas of the striatum, including the medial NAc shell, and more lateral areas project to the NAc core[66–68]. Importantly, the transfection of TH positive cells by the cre-dependent designer receptor construct was highly selective (95.8%), however, only a relatively small proportion of the total TH positive neurons (24.8%) were transfected (Ch 2 Exp. 6). The selectivity of designer receptors for TH positive cells in the current thesis was comparable to similar measures of selectivity in TH::Cre rats[67,131,133,134,143,144,156]. Importantly, the use of designer receptors to modulate the activity of VTA dopamine neurons had been externally demonstrated using c-fos expression, *in vitro* and *in vivo* electrophysiology, and voltammetry to measure dopamine release[134,143]. Thus, the use of cre-dependent viral constructs to express designer receptors on VTA dopamine neurons was highly selective, and capable of modulating neural activity.

Pavlovian conditioning with context alternation can be used to investigate the neural underpinnings of responding to a discrete alcohol CS. This is accomplished by presenting the alcohol CS in the neutral context where responding is not contaminated by an association between the context and alcohol, or a training history involving acquisition and extinction. Under these conditions, systemic administration of the dopamine D2-like antagonist eticlopride attenuated responding to the discrete alcohol CS in the neutral context (Ch 2 Exp. 1). However, this manipulation also attenuated NonCS port entries which may suggest that the broad systemic actions of a dopamine antagonist produced general motor deficits, although there is evidence to discredit this explanation[106,163,164]. Using a chemogenetic approach in TH::Cre rats to inhibit a population of VTA dopamine neurons reduced CS port entries, and circumvented the potential caveat of motor deficit as NonCS port entries were unaffected by this manipulation (Ch 2 Exp. 2). Importantly, CS and NonCS port entries were unaffected by CNO administration in a group of rats devoid of chemogenetic designer receptors, ruling out the explanation of off-target effects contributing to this behaviour (Ch 2 Exp. 3). Thus, VTA dopaminergic activity was necessary for responding to discrete alcohol CS.

Although VTA dopamine neurons were necessary for responding to a discrete alcohol CS, chemogenetic activation of VTA dopamine neurons was not sufficient to elevate responding to a discrete an alcohol CS (Ch 3 Exp. 1). This null effect is difficult to reconcile with the profound effect that chemogenetic activation of VTA dopamine neurons had on feeding behaviour. In the same rats, chemogenetic excitation of VTA dopamine neurons failed to impact responding to a discrete alcohol CS but attenuated feeding behaviour in a subsequent

experiment. This effect on feeding behaviour was pronounced, as consumption during the first two hours, and the entirety, of food access was reduced to ~85.9% and ~78.7% by CNO, and ~82.6% and ~74.6% by clozapine, each respectively and compared to vehicle levels (Ch 3 Exp. 2). This stark reduction in feeding behaviour indicated that chemogenetic activation of VTA dopamine neurons had a profound effect on behaviour, but not an effect that manifested in responding to a discrete alcohol CS.

That VTA dopamine activity is necessary, but not sufficient, for responding to a discrete alcohol CS offers some insight into the mechanism through which dopaminergic activity underpins responding to a discrete alcohol CS. Although activation of VTA dopamine neurons produced an impressive effect on feeding behaviour, this manipulation did not affect responding to a discrete alcohol CS. It could have been predicted that activating VTA dopamine neurons, through disrupting the normal activity of VTA dopamine neurons, would disrupt responding to an alcohol CS, but interestingly CS responding remained stable under vehicle and chemogenetic activation. This stability in CS responding may indicate that the influence of dopaminergic activity over responding to a discrete alcohol CS is gated by another neural circuit. In other words, dopamine activity is necessary for responding to a discrete alcohol CS, but the elevation of this behaviour in an alcohol context may involve the recruitment of additional neural circuits, the activity of which are modulated by dopamine. This hypothesis could be tested by chemogenetically activating VTA dopamine neurons during a test for responding to an alcohol CS in the alcohol context, which was not done in the current thesis because CS responding could reach a ceiling in the alcohol context. A specific circuit hypothesis is that glutamatergic vSub-to-NAc shell inputs, which are necessary for context renewal of alcohol-seeking[70], might be activated by the alcohol context and then modulated by dopamine to elevate CS responding in the alcohol context. This hypothesis supposes that modulation of vSub-to-NAc shell inputs by dopamine when they are not already recruited by relevant contextual stimuli is insufficient to drive responding to a discrete alcohol CS. In summary, VTA dopamine activity was not sufficient to elevate responding to a discrete alcohol CS, however the stability of CS responding during chemogenetic activation of VTA dopamine neurons and under vehicle conditions suggests that another neural circuit might gate the capacity for dopamine to elevate responding to a discrete alcohol CS.

The hypothesis that dopaminergic and glutamatergic activity might act convergently to elevate Pavlovian responding for alcohol, is exciting and testable, however, the failure of

chemogenetic VTA dopamine activity to elevate responding to an alcohol CS can also be explained by technical limitations of the current experiments. The capacity for VTA dopamine neurons to elevate responding to an alcohol CS likely depends on how many neurons are activated, how strongly they are activated, and when they are activated. None of these parameters were manipulated in the current set of experiments, and it stands to reason that a more successful viral transduction of designer receptors, or a more temporally precise stimulation of VTA dopamine neurons might affect responding to a discrete alcohol CS. In support of these alternate explanations are the findings that the capacity for optogenetic stimulation of VTA dopamine neurons to support self-stimulation behaviour depends on the success of the viral infection[133] and the strength of the stimulation parameters[133,225]. As well, dopamine transients in the NAc are time-locked to motivationally salient events[86,165], indicating that their role in controlling behaviour may be temporally delimited. In summary, the lack of an effect of chemogenetic activation of VTA dopamine neurons on responding to an alcohol CS may be attributed to technical limitations of the techniques used in the current thesis.

Dopamine activity in the VTA is necessary, but potentially not sufficient, to elevate responding to a discrete alcohol CS. In the current thesis a dopamine D2-like antagonist and chemogenetic inhibition of VTA dopamine neurons reduced responding to a discrete alcohol CS in a neutral context, the latter without affecting unconditioned behaviour. Using a chemogenetic approach to activate VTA dopamine neurons failed to elevate responding to a discrete alcohol CS which may be attributed to technical limitations of the techniques used. Alternatively, the stability of CS responding under vehicle conditions and chemogenetic activation of VTA dopamine neurons may indicate that the elevation of CS port entries achieved in the alcohol context requires the recruitment of additional circuitries. In sum, there is clear evidence that VTA dopamine neurons are necessary for responding to a discrete alcohol CS. However, the question of whether activation of VTA dopamine neurons or their projections is sufficient to elevate responding to a discrete alcohol CS should be addressed by conducting tests for CS responding in the alcohol and neutral context using techniques that are capable of stimulating neurons in a temporally precise manner and with varying intensity.

The circuitry necessary and sufficient to drive responding to a discrete alcohol CS in different contexts

Despite the strong link between alcohol use disorder and dopamine, pharmacotherapies that act on the dopamine system, show only limited[226,227] if any[228,229] efficacy in aiding recovery

from alcohol use disorder and preventing relapse. One explanation for the lack of efficacious dopamine-based pharmacotherapies, is that separable dopaminergic substrates mediate various aspects of alcohol use disorder and relapse. This view is supported by circuit dissections studies revealing roles for specific projections of various neurotransmitter systems in alcohol-seeking and relapse-like behaviour[70,166]. Further support for the unique roles of separable circuits in responding for alcohol comes from the demonstration in this thesis that chemogenetic VTA-to-NAc core inhibition reduced responding to a discrete alcohol CS irrespective of context, whereas chemogenetic VTA-to-NAc shell inhibition selectively reduced responding to an alcohol CS in an alcohol context (Ch 2 Exps. 4-5). Interestingly, while the dopaminergic VTA-to-NAc shell projection was necessary for responding to a discrete alcohol CS in an alcohol context, chemogenetic activation of this projection did not affect responding to a discrete alcohol CS and increasing glutamatergic activity in the NAc shell reduced CS responding in a context-dependent manner (Ch 3 Exps. 3 & 5). The experiments in this thesis support the hypothesis that distinct neural circuits underlie separable aspects of responding for alcohol and provide clues as to which circuits may be sufficient for driving responses to a discrete alcohol CS in an alcohol context.

An important feature of the experiments in the current thesis was the use of cre-dependent designer receptors constructs in TH::Cre rats to target the projections of VTA dopamine neurons to the NAc core and shell. Designer receptors expressed in the cell bodies of VTA dopamine neurons have been shown to traffic to terminals in the striatum[134,142–145]. However, functional validation of designer receptors expressed on VTA dopamine neurons had focused on cell bodies[134,143] with only one study[134] showing that designer receptors expressed on striatal terminals modulate dopamine release. As such, there was a vacancy in the literature regarding a question critical to this thesis; whether chemogenetic modulation of VTA terminals in the striatum affected the activity of postsynaptic MSNs. It was confirmed by recording electrically-evoked EPSCs in MSNs that were innervated by VTA dopamine terminals expressing inhibitory or excitatory designer receptors, that CNO acting on these designer receptors modulated the activity of NAc core MSNs. Importantly, this bidirectional modulation precludes the concern that CNO produced off-target effects by acting on native receptors[135], or at least confirms that the actions of CNO on designer receptors determines the neural response. Thus, a circuit-specific chemogenetic approach could be used to influence the activity of postsynaptic MSNs targeted by VTA dopamine neurons.

The finding that chemogenetic activation or inhibition of VTA terminals in the NAc core modulated electrically-evoked EPSCs is consistent with the actions of dopamine on corticostriatal terminals in the NAc. Dopamine can act on presynaptic corticostriatal inputs in the NAc to elevate excitatory glutamatergic input onto postsynaptic MSNs[76,170,172–175,183,184,230]. This mechanism is particularly likely in the current thesis because electrical stimulation in the electrophysiology experiments (Ch 2 Exp. 7; Ch 3 Exp. 4) likely recruited corticostriatal inputs to evoke EPSCs. This is further evidenced by the lack of an inhibitory chemogenetic effect in the presence of kynurenic acid, an AMPA and glutamate antagonist (data not shown). Thus, the circuit-specific chemogenetic approach may have manifested behavioural effects *in vivo* through dopaminergic modulation of corticostriatal inputs.

The validated circuit specific approach was used to test the necessity of dopaminergic VTA-to-NAc core and shell projections for responding to a discrete alcohol CS in the neutral and alcohol context. Conducting tests for CS responding in both the alcohol and neutral contexts was critical to dissociate the neural circuits that underpin responding to a discrete alcohol CS and the elevation of this behaviour in an alcohol context. Chemogenetic inhibition of the VTA-to-NAc core circuit, reduced responding to a discrete alcohol CS irrespective of context (Ch 2 Exp. 4). This result recapitulated the reduction in CS responding in a neutral context produced by chemogenetic inhibition of VTA dopamine neurons (Ch 2 Exp. 2). Interestingly, chemogenetic inhibition of the VTA-to-NAc shell projection did not affect CS responding in the neutral context, but reduced CS responding selectively in the alcohol context (Ch 2 Exp. 5). Collectively, these dissociable effects suggest that the dopaminergic VTA-to-NAc core projection supports responding to a discrete alcohol CS, whereas the dopaminergic VTA-to-NAc shell projection supports the elevation of this behaviour in an alcohol context.

Although, the behavioural effects of circuit-specific chemogenetic inhibition were dissociable, there are important caveats that must be considered regarding the use of CNO as a designer ligand[135]. Importantly, it was shown in this thesis that systemically administered CNO and its parent compound clozapine did not affect responding to a discrete alcohol CS in the absence of designer receptors. It has also been shown that CNO microinfusion into the NAc core or shell at the same concentration (3 mM) and volume (0.3 μl) used in the current thesis did not affect responding to a discrete alcohol CS in the alcohol or neutral context in rats without designer receptors[145]. Further, the same CNO microinfusion parameters used here failed to affect motivated behaviours in other studies[146,147]. Another important caveat, that CNO may

diffuse in appreciable concentrations from the NAc core to the shell is unsubstantiated by a study that simultaneously microinfused CNO into the NAc core at the same parameters used in this thesis and collected microdialysis samples in the NAc shell. Using high performance liquid chromatography and mass spectrometry, this study failed to detect CNO in the NAc shell despite achieving a quantitation limit (0.3 nM) lower than has been shown necessary for CNO to activate designer receptors[135]. Thus, the circuit-specific chemogenetic dissociation of VTA-to-NAc core and shell function is unlikely to be attributed to off-target effects of CNO microinfusion or CNO diffusion across NAc subregions.

The dopaminergic VTA-to-NAc core projection may support responding to a discrete alcohol CS through orchestrating behaviour in response to the best predictors of reinforcement. Supporting this idea, observational studies reveal the development of dopamine transients in the NAc core that are time locked to the onset and offset of food-predictive cues[165], and that track closely with the earliest reliable predictor of reinforcement[86]. Also, pairing a discrete CS with optogenetic stimulation of the VTA-to-NAc core projection is sufficient to produce a conditioned response[67]. In the Pavlovian conditioning with context alternation procedure, the discrete CS is the best predictor of alcohol delivery because the alcohol context is present during long periods without alcohol. Thus, the finding that CS responding was reduced by chemogenetic VTA-to-NAc core inhibition irrespective of context, strongly supports the hypothesis that this projection subserves responding to discrete alcohol-predictive cues.

The necessity of dopaminergic VTA-to-NAc shell activity for the elevation of CS responding in the alcohol context, and previous demonstrations that dopaminergic activity in the NAc is elevated drug seeking behaviour[185] suggested that activating this projection might elevate responding to a discrete alcohol CS. Importantly, the excitatory circuit-specific chemogenetic approach had been validated, confirming that CNO acting on excitatory designer receptors on dopaminergic terminals in the NAc could affect the activity of postsynaptic MSNs (Ch 3 Exp. 4). Chemogenetic activation of the VTA-to-NAc shell projection failed to affect responding to a discrete alcohol CS in either the alcohol or neutral context (Ch 3 Exp. 3). This null effect recapitulates the lack of an effect of chemogenetic activation of VTA dopamine neurons on CS responding in the neutral context (Ch 3 Exp. 1). Collectively, chemogenetic activation of VTA dopamine neurons or their projections to the NAc shell failed to affect CS responding.

There are many findings that suggest an interplay between dopaminergic and glutamatergic activity in the NAc shell underpins the elevation of responding to a discrete alcohol CS in an alcohol context. For example, dopamine and glutamate levels in the NAc are interdependent[75–78,191] and elevated during the presentation of alcohol cues[79,195]. Contexts associated with prior drug use can potentiate cocaine-induced elevations in glutamate levels in the NAc[231]. Further, glutamatergic vSub-to-NAc shell inputs are necessary for context renewal of alcohol-seeking[70] and dopaminergic VTA-to-NAc shell inputs are necessary for the elevation of responding to a discrete alcohol CS in an alcohol context (Ch 2 Exp. 5). The most compelling evidence that glutamatergic and dopaminergic activity in the NAc is sufficient to trigger drug seeking behaviour comes from a study showing that dopamine or AMPA microinfused into the NAc reinstates cocaine-seeking[185]. In this study, the capacity for dopamine microinfused into the NAc to reinstate cocaine-seeking was nulled by the co-microinfusion of a glutamate antagonist, whereas the capacity for AMPA to reinstate cocaine-seeking was unaffected by co-microinfusion of a dopamine antagonist. The dependence of dopamine-induced reinstatement of cocaine-seeking upon glutamate activity is consistent with a role for dopaminergic actions on presynaptic corticostriatal inputs to elevate glutamate release onto postsynaptic MSNs in the NAc[76,170,172–175,183,184,230]. If dopaminergic inputs from the VTA are gated by glutamatergic inputs to NAc shell, then exciting dopaminergic inputs in isolation may be insufficient to affect CS responding, which explains the lack of an effect of chemogenetic excitation of VTA dopamine neurons and their inputs to the NAc shell on responding to a discrete alcohol CS.

The hypothesis that glutamatergic vSub-to-NAc shell inputs are recruited by the alcohol context and then modulated by dopaminergic inputs from the VTA to elevate responding to a discrete alcohol CS is unsubstantiated by the lack of an effect of chemogenetic VTA-to-NAc shell activation on CS responding in the alcohol context. Presumably, the vSub-to-NAc shell projection would be recruited by the alcohol context in this test, and thus susceptible to modulation by chemogenetic activation of VTA-to-NAc shell dopaminergic inputs. However, it is possible that CS responding had reached a ceiling in the alcohol context in this experiment and could not be further elevated, although CS port entries had reached higher levels in the alcohol context in other experiments in this thesis. Alternatively, the modulatory actions of VTA dopamine neurons onto corticostriatal inputs may need to be time-locked to CS presentations to elevate CS responding. Indeed, dopamine release in the NAc and NAc shell tracks closely with the onset and offset of motivationally relevant cues[67,86,165]. vSub-to-NAc shell glutamatergic inputs may be tonically activated by the alcohol context, and brief increases in activity in this

projection produced by CS-induced excitation of VTA-to-NAc shell dopamine inputs may serve to elevate responding to the discrete alcohol CS. Thus, an appropriate test of the sufficiency of dopaminergic VTA-to-NAc shell inputs to elevate responses to a discrete alcohol CS would be to use chemogenetics to activate the vSub-to-NAc shell projection during a test for CS responding in the neutral context and optogenetically stimulate the dopaminergic VTA-to-NAc shell projection during CS presentations.

The direct actions of AMPA to excite NAc shell MSNs reduced responding to a discrete alcohol CS specifically in an alcohol context and this finding is inconsistent with the view that convergent vSub- and VTA-to-NAc shell activity underlies elevated responding to an alcohol CS in an alcohol context. Chemogenetic modulation of dopaminergic VTA-to-NAc core inputs bidirectionally regulated the activity of postsynaptic MSNs, which is consistent with dopamine acting on presynaptic corticostriatal terminals to increase glutamate release[76,170,172–175,183,184,230]. Thus, AMPA should excite MSNs, and presumably affect behaviour differently than chemogenetic VTA-to-NAc shell inhibition, which likely reduced the activity of MSNs *in vivo*. Curiously, this was not the case as AMPA microinfused into the NAc shell selectively reduced responding to a discrete alcohol CS in an alcohol context; replicating the behavioural consequence of chemogenetic inhibition of the dopaminergic VTA-to-NAc shell projection. Thus, there appears to be an incompatibility between the opposing neural outcomes of chemogenetic VTA-to-NAc shell inhibition and AMPA microinfusion in the NAc shell, and the similar behavioural outcomes these manipulations produce.

There is a circuit description of NAc shell subnuclei that differ in their connectivity to VTA dopamine neurons and their involvement in motivated behaviour. MSNs located in the medial NAc shell, wherein most of the injector placements in the current thesis were targeted, project onto two separate populations of VTA dopamine neurons: one population projects back to the medial NAc shell and the other projects to the lateral NAc shell[66]. MSNs located in the lateral NAc shell exclusively project onto γ-aminobutyric acid (GABA) interneurons in the VTA, that in turn project onto VTA dopamine neurons that exclusively project back to the lateral shell. Further, VTA dopamine neurons that project to the medial shell express the A subtype of the GABA receptor ($GABA_A$) whereas VTA dopamine neurons that project to the lateral shell express the B subtype GABA ($GABA_B$) receptor. Interestingly, optogenetic stimulation of medial shell MSNs that project to the VTA produces a state of behavioural suppression unless a $GABA_B$ antagonist is microinfused into the VTA, in which case this stimulation supports real-

time place preference. Thus, a medial NAc shell to VTA to lateral NAc shell circuit, when activated, interferes with the expression of motivated behaviour. Differently, optogenetic stimulation of lateral shell MSNs that project to the VTA supports real-time place preference in the absence of a GABA receptor antagonist[66]. Thus, MSNs in the medial and lateral shell differ in their connectivity to VTA dopamine neurons, where those VTA dopamine neurons project to, and the involvement of their respective circuits in motivated behaviour[66].

The separable projections of NAc shell subnuclei explains that chemogenetic inhibition of dopaminergic inputs to the medial NAc shell and AMPA microinfusion in the medial NAc shell target separate circuitries. Specifically, chemogenetic inhibition of VTA-to-NAc shell inputs acts only to reduce dopaminergic activity in medial NAc shell. Conversely, infusing AMPA into the medial NAc shell activates MSNs that project to VTA dopamine neurons that project back onto MSNs in both the medial and lateral NAc shell, ultimately decreasing dopaminergic activity in both of these NAc shell subnuclei. Importantly, activating the projection from the medial NAc shell onto VTA dopamine neurons that project to the lateral NAc shell may suppress motivated behaviour, including responding to a discrete alcohol CS. Thus, using AMPA to manipulate the activity of NAc shell MSNs, through downstream effects, engages dopaminergic projections to both the medial and lateral NAc shell whereas targeting dopaminergic VTA-to-NAc shell projections with chemogenetics is restricted to dopaminergic activity in the medial NAc shell. Thus, the potential for AMPA in the NAc shell to act on a medial NAc shell to VTA to lateral NAc shell circuit and inhibit dopaminergic activity in the lateral NAc shell, may underlie the capacity for AMPA in the NAc shell to selectively reduce responding to an alcohol CS in an alcohol context. This hypothesis is especially plausible because the dopaminergic projection from the VTA to the lateral NAc shell synapses onto inhibitory MSNs that predominantly project onto GABA interneurons in the VTA, which when disinhibited ultimately reduce the activity of VTA dopamine neurons[66].

Conclusion

Context can act in many ways to influence responding to a discrete alcohol CS. A view espoused by Asratyan[10] and other learning theorists[13] was that context is *superordinate* compared to discrete cues because contexts served to signal more information compared to the discrete CS, which mostly instructed the timing of the US. This view was based on the observations that contexts persist before, after and during discrete CSs, and have the capacity to determine many features of the response elicited by a discrete CS. The experiments in this

thesis might support this view, as the alcohol context exhibited robust, and replicable control over responses elicited by a discrete alcohol CS. The control that context exerts over responses elicited by a discrete alcohol cue is particularly relevant for alcohol use disorder, because this control can lead to relapse episodes[1]. The experiments in this thesis show that context powerfully influences responding to a discrete alcohol CS and suggest that a mechanistic understanding of this influence informs the study of alcohol use disorder, relapse, and the development of efficacious therapies.

A key finding from this thesis is that the activity of VTA dopamine neurons and some of their projections are necessary, but appear not sufficient, for responding to a discrete alcohol CS. Chemogenetic inhibition of a population of VTA dopamine neurons reduced responding to a discrete alcohol CS in a neutral context, whereas activation of a similar population of VTA dopamine neurons failed to elevate the same behaviour. At a circuit level, the dopaminergic VTA-to-NAc core projection is necessary for responding to a discrete alcohol CS irrespective of context, whereas the dopaminergic VTA-to-NAc shell projection is necessary for the elevation of this behaviour in an alcohol context. Furthermore, chemogenetic activation of the dopaminergic VTA-to-NAc shell projection did not affect responding to a discrete alcohol CS in the alcohol or neutral context and AMPA microinfused directly into the NAc shell selectively reduced responding to an alcohol CS in an alcohol context. As a summary, dopaminergic projections to the NAc core and shell subserve separable aspects of responding to a discrete alcohol CS in an alcohol context, activating the dopaminergic projection to the NAc shell is not sufficient to elevate responding to a discrete alcohol CS, and glutamatergic shell activity is critical for responding to an alcohol CS in an alcohol context.

There is a potential circuit mechanism that explains the findings in the current thesis and predicts the activity sufficient to elevate responding to a discrete alcohol CS. The proposed mechanism is that dopaminergic inputs to the NAc shell act on corticostriatal inputs from the vSub, which are tonically active in an alcohol context, to increase glutamate release onto postsynaptic MSNs and consequently elevate responding to a discrete alcohol CS. There is evidence that dopaminergic activity in the NAc can modulate the output of corticostriatal terminals[76,170,172–175,183,184,230] that is consistent with the chemogenetic modulation of MSN EPSCs by designer receptors on VTA dopamine terminals in the NAc core in the current thesis (Ch 2 Exp. 7; Ch 3 Exp. 4). Importantly, this circuit mechanism explains why chemogenetic inhibition of VTA-to-NAc shell terminals reduced responding to a discrete alcohol CS in an

alcohol context and chemogenetic activation of the same projection failed elevate CS responding. Specifically, inhibition of the dopaminergic VTA-to-NAc shell projection interferes with CS-induced increases in VTA-to-NAc shell dopaminergic activity which modulates vSub-to-NAc shell activity. However, the dopaminergic VTA-to-NAc shell activity sufficient to elevate responding to a discrete alcohol CS must occur onto a tonically active vSub-to-NAc shell projection and likely in a temporally precise manner during CS presentations; the chemogenetic approach lacks this temporal specificity and the vSub-to-NAc shell projection may not be active in a neutral context. Lastly, this circuit mechanism is consistent with a role for a GABAergic projection from the medial NAc shell to a population of VTA dopamine neurons that project to the lateral NAc shell in suppressing motivated behaviour. AMPA microinfused into the medial NAc shell would drive inhibition onto this subpopulation of VTA dopamine neurons that project to the lateral NAc shell, and inhibit responding to a discrete alcohol CS[66]. A critical test of this proposed circuit mechanism is to use chemogenetics to activate the vSub-to-NAc shell projection during a test for CS responding in the neutral context and optogenetically stimulate the VTA-to-NAc shell projection during CS presentations. If the elevation of responding to a discrete alcohol CS in an alcohol context is caused by the tonic activation of vSub-to-NAc shell inputs which are modulated by CS-induced activation of the dopaminergic VTA-to-NAc shell projection, then this circuit manipulation should elevate responding to a discrete alcohol CS in a neutral context.

The role of the dopamine system as a critical substrate in substance use disorder has long been postulated[197], and remains of interest today[200], however dopamine-based pharmacotherapies to treat alcohol use disorder and relapse have largely been ineffective[160,162,228,229]. The current thesis suggests three potential explanations for the limited efficacy of dopamine-based pharmacotherapies in treating alcohol use disorder. First, the types of environmental cues, namely discrete cues and contexts, that have the capacity to generate an urge to drink[3] or precipitate a relapse episode[1], are varied, and when they occur in combination exert a particularly powerful control over responding for alcohol. Second, discrete alcohol cues and contexts engage separate and specific dopamine circuits to control behaviour, and the incidental targeting of these circuits by broad acting pharmacological agents, is ineffective at mitigating relapse. Thirdly, dopamine circuits interact with and may be gated by other neurotransmitter systems to elevate responding for alcohol in alcohol associated contexts and thus efficacious pharmacotherapies must target multiple neurotransmitter systems or a critical interface between these systems. Collectively, the findings from this thesis encourage a

more nuanced investigation[182] of neural circuitries that underpin dissociable influences of discrete cues and contexts on responding for alcohol to further the endeavour of developing efficacious therapies for alcohol use disorder.

References

1. Marlatt, G. A. Taxonomy of high-risk situations for alcohol relapse: evolution and development of a cognitive-behavioral model. *Addiction* **91**, S37-49 (1996).
2. Ludwig, A. M., Wikler, A. & Stark, L. H. The first drink: Psychobiological aspects of craving. *Arch. Gen. Psychiatry* **30**, 539–47 (1974).
3. Ludwig, A. M. Pavlov's 'bells' and alcohol craving. *Addict. Behav.* **11**, 87–91 (1986).
4. Ludwig, A. M. & Wikler, A. 'Craving' and relapse to drink. *Q. J. Stud. Alcohol* **35**, 108–30 (1974).
5. McCusker, C. G. & Brown, K. Alcohol-predictive cues enhance tolerance to and precipitate '"craving"' for alcohol in social drinkers. *J. Stud. Alcohol* **51**, 494–499 (1990).
6. Pavlov, I. P. *Conditoined Reflexes*. *An investigation of the physiological activity of the cerebral cortex* (Oxford University Press, 1927). doi:10.1002/9781119196624.ch6.
7. Pavlovian Society. https://campus.albion.edu/pavlovian/.
8. Bitterman, M. E. Classical Conditioning Since Pavlov. *Rev. Gen. Psychol.* **10**, 365–376 (2006).
9. Windholz, G. Ivan P Pavlov An overview of his life and work. *Am. Psychol.* **52**, 941–946 (1997).
10. Asratyan, E. A. The physiology of the conditioned reflex. in *Compensatory adaptations, reflex activity, and the brain* (ed. Corson, S. A.) 121–147 (Pergamon Press, 1965).
11. Kimmel, H. D. & Ray, R. L. Transswitching: Conditioning with tonic and phasic stimuli. *J. Exp. Psychol. Gen.* **107**, 187–205 (1978).
12. Lachnit, H. Transswitching and contextual conditioning. *Integr. Physiol. Behav. Sci.* **21**, 160–172 (1986).
13. Nadel, L. & Willner, J. Context and conditioning: A place for space. *Physiol. Psychol.* **8**, 218–228 (1980).
14. Brandon, T. H., Vidrine, J. I. & Litvin, E. B. Relapse and Relapse Prevention. *Annu. Rev. Clin. Psychol.* **3**, 257–284 (2007).
15. Sinha, R. New findings on biological factors predicting addiction relapse vulnerability. *Curr. Psychiatry Rep.* **13**, 398–405 (2011).
16. Billings, A. G., Weiner, S., Kessler, M. & Gomberg, C. A. Drinking behavior in laboratory and barroom settings. *J. Stud. Alcohol* **37**, 85–89 (1976).
17. Wigmore, S. W. & Hinson, R. E. The influence of setting on consumption in the balanced placebo design. *Br. J. Addict.* **86**, 205–215 (1991).

18. Strickler, D. P., Dobbs, S. D. & Maxwell, W. A. The influence of setting on drinking behaviors: The laboratory vs the barroom. *Addict. Behav.* **4**, 339–344 (1979).
19. Drummond, D. C. & Glautier, S. A Controlled Trial of Cue Exposure Treatment in Alcohol Dependence. *J. Consult. Clin. Psychol.* **62**, 809–817 (1994).
20. Conklin, C. A. & Tiffany, S. T. Applying extinction research and theory to cue-exposure addiction treatments. *Addiction* **97**, 155–167 (2002).
21. Hodgson, R., Rankin, H. & Stockwell, T. Alcohol dependence and the priming effect. *Behav. Res. Ther.* **17**, 379–387 (1979).
22. Rankin, H., Hodgson, R. & Stockwell, T. Cue exposure and response prevention with alcoholics: A controlled trial. *Behav. Res. Ther.* **21**, 435–446 (1983).
23. Stewart, J., de Wit, H. & Eikelboom, R. Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants. *Psychol. Rev.* **91**, 251–268 (1984).
24. Davis, W. M. & Smith, S. G. Role of conditioned reinforcers in the initiation, maintenance and extinction of drug-seeking behavior. *The Pavlovian journal of biological science* vol. 11 222–236 (1976).
25. Shaham, Y., Shalev, U., Lu, L., de Wit, H. & Stewart, J. The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology (Berl).* **168**, 3–20 (2003).
26. Stewart, J. & de Wit, H. Reinstatement of drug-taking behavior as a method os assessing incentive motivational properties of drugs. in *Methods of assessing the reinforcing properties of abused drugs* (ed. Bozarth, M. A.) 211–227 (Springer-Verlag, 1987).
27. de Wit, H. & Stewart, J. Reinstatement of cocaine-reinforced responding in the rat. *Psychopharmacology (Berl).* **75**, 134–143 (1981).
28. de Wit, H. & Stewart, J. Drug reinstatement of heroin-reinforced responding in the rat. *Psychopharmacology (Berl).* **79**, 29–31 (1983).
29. Liu, X. & Weiss, F. Additive effect of stress and drug cues on reinstatement of ethanol seeking: Exacerbation by history of dependence and role of concurrent activation of corticotropin-releasing factor and opioid mechanisms. *J. Neurosci.* **22**, 7856–7861 (2002).
30. Nie, H. & Janak, P. H. Comparison of reinstatement of ethanol- and sucrose-seeking by conditioned stimuli and priming injections of allopregnanolone after extinction in rats. *Psychopharmacology (Berl).* **168**, 222–228 (2003).
31. Bienkowski, P., Koros, E., Kostowski, W. & Bogucka-Bonikowska, A. Reinstatement of ethanol seeking in rats: Behavioral analysis. *Pharmacol. Biochem. Behav.* **66**, 123–128

(2000).
32. Tsiang, M. T. & Janak, P. H. Alcohol seeking in C57BL/6 mice induced by conditioned cues and contexts in the extinction-reinstatement model. *Alcohol* **38**, 81–8 (2006).
33. Glautier, S., Drummond, D. C. & Remington, B. Different drink cues elicit different physiological responses in non-dependent drinkers. *Psychopharmacology (Berl).* **106**, 550–554 (1992).
34. Glautier, S., Drummond, C. & Remington, B. Alcohol as an unconditioned stimulus in human classical conditioning. *Psychopharmacology (Berl).* **116**, 360–368 (1994).
35. Stewart, J. Reinforcing effects of light as a function of intensity and reinforcement schedule. *J. Comp. Physiol. Psychol.* **53**, 187–193 (1960).
36. Caggiula, A. R. *et al.* Environmental stimuli promote the acquisition of nicotine self-administration in rats. *Psychopharmacology (Berl).* **163**, 230–237 (2002).
37. Olsen, C. M. & Winder, D. G. Stimulus dynamics increase the self-administration of compound visual and auditory stimuli. *Neurosci. Lett.* **511**, 8–11 (2012).
38. Valyear, M. D., Villaruel, F. R. & Chaudhri, N. Alcohol-seeking and relapse: A focus on incentive salience and contextual conditioning. *Behav. Processes* **141**, 26–32 (2017).
39. Lau-Barraco, C. & Dunn, M. E. Environmental context effects on alcohol cognitions and immediate alcohol consumption. *Addict. Res. Theory* **17**, 306–314 (2009).
40. Monk, R. L., Qureshi, A. W., Mcneill, A., Erskine-shaw, M. & Heim, D. Perfect for a Gin and Tonic: How Context Drives Consumption Within a Modified Bogus Taste Test. *Alcohol Alcohol.* **53**, 228–234 (2018).
41. Childs, E. & de Wit, H. Alcohol-induced place conditioning in moderate social drinkers. *Addiction* **111**, 2157–2165 (2016).
42. Mayo, L. M. *et al.* Conditioned preference to a methamphetamine-associated contextual cue in humans. *Neuropsychopharmacology* **38**, 921–9 (2013).
43. Bouton, M. E. & Bolles, R. C. Contextual control of the extinction of conditioned fear. *Learn. Motiv.* **10**, 445–466 (1979).
44. Bouton, M. E. & Bolles, R. C. Role of conditioned contextual stimuli in reinstatement of extinguished fear. *J. Exp. Psychol. Anim. Behav. Process.* **5**, 368–378 (1979).
45. Bouton, M. E. What ever happened to behavior anyway? in *Learning and behavior: A contemporary synthesis* 160–69 (Oxford University Press, 2007).
46. Crombag, H. S. & Shaham, Y. Renewal of drug seeking by contextual cues after prolonged extinction in rats. *Behav. Neurosci.* **116**, 169–173 (2002).
47. Crombag, H. S., Grimm, J. W. & Shaham, Y. Effect of dopamine receptor antagonists on

renewal of cocaine seeking by reexsposure to drug-associated contextual cues. *Neuropsychopharmacology* **27**, 1008–1015 (2002).

48. Crombag, H. S., Bossert, J. M., Koya, E. & Shaham, Y. Context-induced relapse to drug seeking: a review. *Philos. Trans. R. Soc. London* **363**, 3233–43 (2008).
49. Marchant, N. J., Campbell, E. J., Pelloux, Y., Bossert, J. M. & Shaham, Y. Context-induced relapse after extinction versus punishment: similarities and differences. *Psychopharmacology (Berl).* **236**, 439–448 (2019).
50. Venniro, M., Caprioli, D. & Shaham, Y. *Animal models of drug relapse and craving: From drug priming-induced reinstatement to incubation of craving after voluntary abstinence*. *Progress in Brain Research* vol. 224 (Elsevier B.V., 2016).
51. Burattini, C., Gill, T. M., Aicardi, G. & Janak, P. H. The ethanol self-administration context as a reinstatement cue: Acute effects of naltrexone. *Neuroscience* **139**, 877–887 (2006).
52. Zironi, I., Burattini, C., Aicardi, G. & Janak, P. H. Context is a trigger for relapse to alcohol. *Behav. Brain Res.* **167**, 150–155 (2006).
53. Hamlin, A. S., Newby, J. & McNally, G. P. The neural correlates and role of D1 dopamine receptors in renewal of extinguished alcohol-seeking. *Neuroscience* **146**, 525–536 (2007).
54. Collins, B. N. & Brandon, T. H. Effects of extinction context and retrieval cues on alcohol cue reactivity amoung nonalcoholic drinkers. *J. Consult. Clin. Psychol.* **70**, 390–97 (2002).
55. Chaudhri, N., Sahuque, L. L. & Janak, P. H. Context-induced relapse of conditioned behavioral responding to ethanol cues in rats. *Biol. Psychiatry* **64**, 203–10 (2008).
56. Chaudhri, N., Sahuque, L. L., Schairer, W. W. & Janak, P. H. Separable roles of the nucleus accumbens core and shell in context- and cue-induced alcohol-seeking. *Neuropsychopharmacology* **35**, 783–91 (2010).
57. Sciascia, J. M., Reese, R. M., Janak, P. H. & Chaudhri, N. Alcohol-seeking triggered by discrete Pavlovian cues is invigorated by alcohol contexts and mediated by glutamate signaling in the basolateral amygdala. *Neuropsychopharmacology* 1–12 (2015) doi:10.1038/npp.2015.130.
58. Swanson, L. W. The projections of the ventral tegmental area and adjacent regions: A combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res. Bull.* **9**, 321–353 (1982).
59. Phillipson, O. T. & Griffiths, A. C. The topographic order of inputs to nucleus accumbens in the rat. *Neuroscience* **16**, 275–296 (1985).

60. Janak, P. H. & Tye, K. M. From circuits to behaviour in the amygdala. *Nature* **517**, 284–292 (2015).
61. Mcdonald, A. J. Organization of amygdaloid projections to the prefrontal cortex and associated striatum in the rat. *Neuroscience* **44**, 1–14 (1991).
62. Brog, J. S., Salyapongse, A., Deutch, A. Y. & Zahm, D. S. The patterns of afferent innervation of the core and shell in the “Accumbens” part of the rat ventral striatum: Immunohistochemical detection of retrogradely transported fluoro-gold. *J. Comp. Neurol.* **338**, 255–278 (1993).
63. Britt, J. P. *et al.* Synaptic and Behavioral Profile of Multiple Glutamatergic Inputs to the Nucleus Accumbens. *Neuron* **76**, 790–803 (2012).
64. Reed, S. J. *et al.* Coordinated Reductions in Excitatory Input to the Nucleus Accumbens Underlie Food Consumption. *Neuron* **99**, 1260-1273.e4 (2018).
65. Groenewegen, H. J., der Zee, E. V. Van, te Kortschot, A. & Witter, M. P. Organization of the projections from the subiculum to the ventral striatum in the rat. A study using anterograde transport of Phaseolus vulgaris leucoagglutinin. *Neuroscience* **23**, 103–120 (1987).
66. Yang, H. *et al.* Nucleus accumbens subnuclei regulate motivated behavior via direct inhibition and disinhibition of VTA dopamine subpopulations. *Neuron* **97**, 434-449.e4 (2018).
67. Saunders, B. T., Richard, J. M., Margolis, E. B. & Janak, P. H. Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties. *Nat. Neurosci.* **21**, 1 (2018).
68. Ikemoto, S. Dopamine reward circuitry: Two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res. Rev.* **56**, 27–78 (2007).
69. Chaudhri, N., Woods, C. A., Sahuque, L. L., Gill, T. M. & Janak, P. H. Unilateral inactivation of the basolateral amygdala attenuates context-induced renewal of Pavlovian-conditioned alcohol-seeking. *Eur. J. Neurosci.* **38**, 2751–2761 (2013).
70. Marchant, N. J. *et al.* Role of ventral subiculum in context-Induced relapse to alcohol seeking after punishment-imposed abstinence. *J. Neurosci.* **36**, 3281–3294 (2016).
71. Chaudhri, N., Sahuque, L. L. & Janak, P. H. Ethanol seeking triggered by environmental context is attenuated by blocking dopamine D1 receptors in the nucleus accumbens core and shell in rats. *Psychopharmacology (Berl).* **207**, 303–314 (2009).
72. Sciascia, J. M., Mendoza, J. & Chaudhri, N. Blocking dopamine d1-like receptors attenuates context-induced renewal of pavlovian-conditioned alcohol-seeking in rats.

Alcohol. Clin. Exp. Res. **38**, 418–27 (2014).

73. Millan, E. Z., Reese, R. M., Grossman, C. D., Chaudhri, N. & Janak, P. H. Nucleus accumbens and posterior amygdala mediate cue-triggered alcohol seeking and suppress behavior during the omission of alcohol-predictive cues. *Neuropsychopharmacology* 1–11 (2015) doi:10.1038/npp.2015.102.
74. Sesack, S. R. & Pickel, V. M. In the rat medial nucleus accumbens, hippocampal and catecholaminergic terminals converge on spiny neurons and are in apposition to each other. *Brain Res.* **527**, 266–279 (1990).
75. Xue, C.-J., Ng, J. P., Li, Y. & Wolf, M. E. Acute and Repeated Systemic Amphetamine Administration: Effects on Extracellular Glutamate, Aspartate, and Serine Levels in Rat Ventral Tegmental Area and Nucleus Accumbens. *J. Neurochem.* **67**, 352–363 (2002).
76. Smith, J. A., Qiu Mo, Guo, H., Kunko, P. M. & Robinson, S. E. Cocaine increases extraneuronal levels of aspartate and glutamate in the nucleus accumbens. *Brain Res.* **683**, 264–269 (1995).
77. Dalia, A., Uretsky, N. J. & Wallace, L. J. Dopaminergic agonists administered into the nucleus accumbens: Effects on extracellular glutamate and on locomotor activity. *Brain Res.* **788**, 111–117 (1998).
78. Svensson, L., Zhang, J., Johannessen, K. & Engel, J. A. Effect of local infusion of glutamate analogues into the nucleus accumbens of rats: an electrochemical and behavioural study. *Brain Res.* **643**, 155–161 (1994).
79. Katner, S. N. & Weiss, F. Ethanol-associated olfactory stimuli reinstate ethanol-seeking behavior after extinction and modify extracellular dopamine levels in the nucleus accumbens. *Alcohol. Clin. Exp. Res.* **23**, 1751–1760 (1999).
80. Yim, H. J., Schallert, T., Randall, P. K. & Gonzales, R. A. Comparison of local and systemic ethanol effects on extracellular dopamine concentration in rat nucleus accumbens by microdialysis. *Alcohol. Clin. Exp. Res.* **22**, 367–74 (1998).
81. Yoder, K. K. *et al.* When what you see isn't what you get: Alcohol cues, alcohol administration, prediction error, and human striatal dopamine. *Alcohol. Clin. Exp. Res.* **33**, 139–149 (2009).
82. Boileau, I. *et al.* Alcohol promotes dopamine release in the human nucleus accumbens. *Synapse* **49**, 226–231 (2003).
83. Volkow, N. D. *et al.* Profound decreases in dopamine release in striatum in detoxified alcoholics: Possible orbitofrontal involvement. *J. Neurosci.* **27**, 12700–12706 (2007).
84. Volkow, N. D. *et al.* Decreases in dopamine receptors but not in dopamine transporters in

alcoholics. *Alcohol. Clin. Exp. Res.* **20**, 1594–1598 (1996).

85. Liu, X. & Weiss, F. Reversal of ethanol-seeking behavior by D1 and D2 antagonists in an animal model of relapse: differences in antagonist potency in previously ethanol-dependent versus nondependent rats. *J. Pharmacol. Exp. Ther.* **300**, 882–9 (2002).
86. Saddoris, M. P., Cacciapaglia, F., Wightman, R. M. & Carelli, R. M. Differential dopamine release dynamics in the nucleus accumbens core and shell reveal complementary signals for error prediction and incentive motivation. *J. Neurosci.* **35**, 11572–82 (2015).
87. Cacciapaglia, F., Saddoris, M. P., Wightman, R. M. & Carelli, R. M. Differential dopamine release dynamics in the nucleus accumbens core and shell track distinct aspects of goal-directed behavior for sucrose. *Neuropsychopharmacology* **62**, 2050–56 (2012).
88. Lett, B. T. Repeated exposures intensify rather than diminish the rewarding effects of amphetamine, morphine, and cocaine. *Psychopharmacology (Berl).* **98**, 357–362 (1989).
89. Mitchell, J. B. & Stewart, J. Facilitation of sexual behaviors in the male rat in the presence of stimuli previously paired with systemic injectinos of morphine. *Pharmacol. Biochem. Behav.* **35**, 643–650 (1990).
90. Mitchell, J. B. & Stewart, J. Facilitation of sexual behaviors in the male rat associated with Intra-VTA injections of opiates. *Pharmacol. Biochem. Behav.* **35**, 643–650 (1990).
91. Stewart, J. & Vezina, P. Environment-specific enhancement of the hyperactivity induced by systemic or intra-VTA morphine injections in rats preexposed to amphetamine. *Psychobiology* **15**, 144–153 (1987).
92. Vezina, P. & Stewart, J. Conditioning and place-specific sensitization of increases in activity induced by morphine in the VTA. *Pharmacol. Biochem. Behav.* **20**, 925–934 (1984).
93. Vezina, P., Giovino, A. A., Wise, R. A. & Stewart, J. Environment-specific cross-sensitization between the locomotor activating effects of morphine and amphetamine. *Pharmacol. Biochem. Behav.* **32**, 581–584 (1989).
94. Vezina, P. & Leyton, M. Conditioned cues and the expression of stimulant sensitization in animals and humans. *Neuropharmacology* **56**, 160–168 (2009).
95. Bossert, J. M., Poles, G. C., Wihbey, K. A., Koya, E. & Shaham, Y. Differential effects of blockade of dopamine D1-family receptors in nucleus accumbens core or shell on reinstatement of heroin seeking induced by contextual and discrete cues. *J. Neurosci.* **27**, 12655–12663 (2007).
96. Ito, R., Robbins, T. W. & Everitt, B. J. Differential control over cocaine-seeking behavior by nucleus accumbens core and shell. *Nat. Neurosci.* **7**, 389–97 (2004).

97. Ito, R., Dalley, J. W., Howes, S. R., Robbins, T. W. & Everitt, B. J. Dissociation in Conditioned Dopamine Release in the Nucleus Accumbens Core and Shell in Response to Cocaine Cues and during Cocaine-Seeking Behavior in Rats. *J. Neurosci.* **20**, 7489–7495 (2000).
98. Bechtholt, A. J. & Cunningham, C. L. Ethanol-induced conditioned place preference is expressed through a ventral tegmental area dependent mechanism. *Behav. Neurosci.* **119**, 213–223 (2005).
99. Pina, M. M. & Cunningham, C. L. Involvement of ventral tegmental area ionotropic glutamate receptors in the expression of ethanol-induced conditioned place preference. *Behav. Brain Res.* **313**, 23–29 (2016).
100. Risinger, F. O., Malott, D. H., Prather, L. K., Niehus, D. R. & Cunningham, C. L. Motivational properties of ethanol in mice selectively bred for ethanol-induced locomotor differences. *Psychopharmacology (Berl).* **116**, 207–216 (1994).
101. Ciccocioppo, R., Panocka, I., Froldi, R., Quitadamo, E. & Massi, M. Ethanol induces conditioned place preference in genetically selected alcohol-preferring rats. *Psychopharmacology (Berl).* **141**, 235–241 (1999).
102. Nentwig, T. B., Myers, K. P. & Grisel, J. E. Initial subjective reward to alcohol in Sprague-Dawley rats. *Alcohol* **58**, 19–22 (2017).
103. Matsumoto, H., Tian, J. & Uchida, N. Midbrain dopamine neurons signal aversion in a reward-context-dependent manner. *Elife* 1–24 (2016) doi:10.7554/eLife.17328.
104. Remedios, J., Woods, C., Tardif, C., Janak, P. H. & Chaudhri, N. Pavlovian-conditioned alcohol-seeking behavior in rats is invigorated by the interaction between discrete and contextual alcohol cues: implications for relapse. *Brain Behav.* **4**, 278–89 (2014).
105. Janak, P. H. & Chaudhri, N. The potent effect of environmental context on relapse to alcohol-seeking after extinction. *Open Addict. J.* **1**, 76–87 (2010).
106. Sparks, L. M., Sciascia, J. M., Ayorech, Z. & Chaudhri, N. Vendor differences in alcohol consumption and the contribution of dopamine receptors to Pavlovian-conditioned alcohol-seeking in Long-Evans rats. *Psychopharmacology (Berl).* **231**, 753–64 (2014).
107. Davis, M. Sensitization of the rat startle response by noise. *Journal of comparative and physiological psychology* vol. 87 571–581 (1974).
108. Chaudhri, N., Sahuque, L. L., Cone, J. J. & Janak, P. H. Reinstated ethanol-seeking in rats is modulated by environmental context and requires the nucleus accumbens core. *Eur. J. Neurosci.* **28**, 2288–98 (2008).
109. Lacroix, F., Pettorelli, A., Maddux, J. N., Heidari-Jam, A. & Chaudhri, N. Varenicline

reduces context-induced relapse to alcohol-seeking through actions in the nucleus accumbens. *Neuropsychopharmacology* 1–12 (2016) doi:10.1038/npp.2016.254.

110. Conklin, C. A., Robin, N., Perkins, K. A., Salkeld, R. P. & McClernon, F. J. Proximal versus distal cues to smoke: the effects of environments on smokers' cue-reactivity. *Exp. Clin. Psychopharmacol.* **16**, 207–214 (2008).
111. Rescorla, R. A. Inhibition of Delay in Pavlovian Fear Conditioning. *J. Comp. Physiol. Psychol.* **64**, 114–120 (1967).
112. Delamater, A. R. & Holland, P. C. The Influence of CS-US Interval on Several Different Indices of Learning in Appetitive Conditioning. *J. Exp. Psychol. Anim. Behav. Process.* **34**, 202–222 (2008).
113. Balsam, P. D., Drew, M. R. & Yang, C. Timing at the start of associative learning. *Learn. Motiv.* **33**, 141–155 (2002).
114. Pinel, J. P. & Huang, E. Effects of periodic withdrawal on ethanol and saccharin selection in rats. *Physiol. Behav.* **16**, 693–8 (1976).
115. Wayner, M. J. & Fraley, S. Enhancement of the consumption of acclimated sapid solutions following periodic and prolonged withdrawal. *Physiol. Behav.* **9**, 463–74 (1972).
116. Wayner, M. J. *et al.* A new factor affecting the consumption of ethyl alcohol and other sapid fluids. *Physiol. Behav.* **8**, 345–62 (1972).
117. Amit, Z., Stern, M. H. & Wise, R. A. Alcohol preference in the laboratory rat induced by hypothalamic stimulation. *Psychopharmacologia* **17**, 367–77 (1970).
118. Wise, R. A. Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacologia* **29**, 203–10 (1973).
119. Simms, J. A. *et al.* Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcohol. Clin. Exp. Res.* **32**, 1816–23 (2008).
120. Villaruel, F. R. & Chaudhri, N. Individual Differences in the Attribution of Incentive Salience to a Pavlovian Alcohol Cue. *Front. Behav. Neurosci.* **10**, 1–13 (2016).
121. Robinson, T. E. & Flagel, S. B. Dissociating the predictive and incentive motivational properties of reward-related cues through the study of individual differences. *Biol. Psychiatry* **65**, 869–73 (2009).
122. Flagel, S. B. *et al.* A selective role for dopamine in stimulus-reward learning. *Nature* **469**, 53–7 (2011).
123. Robinson, T. E. & Berridge, K. C. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res. Rev.* **18**, 247–291 (1993).
124. Robinson, T. E. & Berridge, K. C. Addiction. *Annu. Rev. Psychol.* **54**, 25–53 (2003).

125. Flagel, S. B., Akil, H. & Robinson, T. E. Individual differences in the attribution of incentive salience to reward-related cues: Implications for addiction. *Neuropharmacology* **56**, 139–148 (2009).
126. Shnitko, T. A. & Robinson, D. L. Regional variation in phasic dopamine release during alcohol and sucrose self-administration in rats. *ACS Chem. Neurosci.* **6**, 147–154 (2015).
127. Mahler, S. V *et al.* Chemogenetic Manipulations of Ventral Tegmental Area Dopamine Neurons Reveal Multifaceted Roles in Cocaine Abuse. *J. Neurosci.* **39**, 503–518 (2018).
128. Ito, R., Robbins, T. W., Pennartz, C. M. & Everitt, B. J. Functional interaction between the hippocampus and nucleus accumbens shell is necessary for the acquisition of appetitive spatial context conditioning. *J. Neurosci.* **28**, 6950–9 (2008).
129. Barker, J. M. *et al.* Corticostriatal circuitry and habitual ethanol seeking. *Alcohol* **49**, 817–824 (2015).
130. Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S. & Roth, B. L. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 5163–5168 (2007).
131. Mahler, S. V *et al.* Designer receptors show role for ventral pallidum input to ventral tegmental area in cocaine seeking. *Nat. Neurosci.* **17**, 577–85 (2014).
132. Sohal, V. S., Zhang, F., Yizhar, O. & Deisseroth, K. Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. *Nature* **459**, 698–702 (2009).
133. Witten, I. B. *et al.* Recombinase-driver rat lines: Tools, techniques, and optogenetic application to dopamine-mediated reinforcement. *Neuron* **72**, 721–733 (2011).
134. Mahler, S. V *et al.* Chemogenetic manipulations of ventral tegmental area dopamine neurons reveal multifaceted roles in cocaine abuse. *J. Neurosci.* **39**, 503–518 (2019).
135. Gomez, J. L. *et al.* Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science (80-.).* **357**, 503–507 (2017).
136. Maclaren, D. A. A. *et al.* Clozapine-n-oxide administration produces behavioral effects in Long-Evans rats - implications for designing DREADD experiments CNO converts into bioactive compounds in rats. *eNeuro* **10**, 219–16 (2016).
137. Thompson, K. J. *et al.* Dreadd Agonist 21 (C21) Is an Effective Agonist for Muscarnic-Based Dreadds in Vitro and in Vivo. *ACS Pharmacol. Transl. Sci.* **21**, (2018).
138. Mahler, S. V & Aston-Jones, G. CNO Evil? Considerations for the Use of DREADDs in Behavioral Neuroscience. *Neuropsychopharmacology* 1–3 (2018) doi:10.1038/npp.2017.299.
139. Jendryka, M. *et al.* Pharmacokinetic and pharmacodynamic actions of clozapine-N-oxide,

clozapine, and compound 21 in DREADD-based chemogenetics in mice. *Sci. Rep.* **9**, 1–14 (2019).

140. Bouton, M. E. Context, Ambiguity, and Unlearning: Sources of Relapse after Behavioral Extinction Four Mechanisms of Relapse. *Biol. Psychiatry* **52**, 976–986 (2002).
141. Todd, T. P. Mechanisms of Renewal After the Extinction of Instrumental Behavior. *J. Exp. Psychol.* **39**, 193–207 (2013).
142. Halbout, B. *et al.* Mesolimbic dopamine projections mediate cue-motivated reward seeking but not reward retrieval in rats. *Elife* **8**, 1–21 (2019).
143. Boekhoudt, L. *et al.* Chemogenetic activation of dopamine neurons in the ventral tegmental area, but not substantia nigra, induces hyperactivity in rats. *Eur. Neuropsychopharmacol.* **26**, 1784–1793 (2016).
144. Boekhoudt, L. *et al.* Chemogenetic Activation of Midbrain Dopamine Neurons Affects Attention, but not Impulsivity, in the Five-Choice Serial Reaction Time-Task in Rats. *Neuropsychopharmacology* **42**, 1–11 (2016).
145. Valyear, M. D. *et al.* Dissociable mesolimbic dopamine circuits control responding triggered by alcohol-predictive discrete cues and contexts. *Nat. Commun.* **11**, 1–14 (2020).
146. Hsu, T. M. *et al.* A hippocampus to prefrontal cortex neural pathway inhibits food motivation through glucagon-like peptide-1 signaling. *Mol. Psychiatry* **23**, 1555–1565 (2018).
147. Companion, M. A. & Thiele, T. E. Assessment of ventral tegmental area-projecting GABAergic neurons from the bed nucleus of the stria terminalis in modulating binge-like ethanol intake. *Eur. J. Neurosci.* **48**, 3335–3343 (2018).
148. Stachniak, T. J., Ghosh, A. & Sternson, S. M. Chemogenetic synaptic silencing of neural circuits localizes a hypothalamus→midbrain pathway for feeding behavior. *Neuron* **82**, 797–808 (2014).
149. Meredith, G. E. & Totterdell, S. Microcircuits in nucleus accumbens' shell and core involved in cognition and reward. *Psychobiology* **27**, 165–186 (1999).
150. Chang, H. T. & Kitai, S. T. Projection neurons of the nucleus accumbens: an intracellular labeling study. *Brain Res.* **347**, 112–116 (1985).
151. Hedreen, J. C. Neurons of the nucleus accumbens and other striatal regions in rats. in *The neurobiology of the nucleus accumbens* 82–96 (Haer Institute for Electrophysiological Research, 1981).
152. Pennartz, C. M. A., Dolleman-Van der Weel, M. J. & Lpoes da Silva, F. H. Differential

memebrane porperties and dopamine effects in the shell and core of the rat nucleus accumbens studied in vitro. *Neurosci. Lett* **136**, 109–112 (1992).

153. Meredith, G. E., Pennartz, C. M. A. & Groenewegen, H. J. The cellular framework for chemical signalling in the nucleus accumbens. *Progress in Brain Research* vol. 99 3–24 (1993).
154. Hjelmstad, G. O. Dopamine excites nucleus accumbens neurons through the differential modulation of glutamate and GABA release. *J. Neurosci.* **24**, 8621–8628 (2004).
155. Mahler, S. V. *et al.* Chemogenetic manipulations of ventral tegmental area dopamine neurons reveal multifaceted roles in cocaine abuse. *bioRxiv* 246595 (2018) doi:10.1101/246595.
156. Sharpe, M. J. *et al.* Dopamine transients are sufficient and necessary for acquisition of model-based associations. *Nat. Neurosci.* **20**, 735–742 (2017).
157. Boekhoudt, L. *et al.* Does activation of midbrain dopamine neurons promote or reduce feeding? *Int. J. Obes.* **41**, 1131–1140 (2017).
158. Pennartz, C. M. A. & Kitai, S. T. Hippocampal inputs to identified neurons in an in vitro slice preparation of the rat nucleus accumbens: evidence for feed-forward inhibition. *J. Neurosci.* **17**, 2838–2847 (1991).
159. O'Donnell, P. & Grace, A. A. Dopaminergic reduction of excitability in nucleus accumbens neurons recorded in vitro. *Neuropsychopharmacology* vol. 15 87–97 (1996).
160. Jayaram-Lindström, N., Ericson, M., Steensland, P. & Jerlhag, E. Dopamine and alcohol dependence: from bench to clinic. in *Recent advances in drug addiction research and clinical applications* 82–114 (2012). doi:10.1016/j.colsurfa.2011.12.014.
161. Koob, G. F. & Volkow, N. D. Neurocircuitry of Addiction. *Neuropsychopharmacology* **35**, 217–238 (2010).
162. Swift, R. Medications Acting on the Dopaminergic System in the Treatment of Alcoholic Patients. *Curr. Pharm. Des.* **16**, 2118–2125 (2010).
163. Khoo, S. Y.-S., Uhrig, A. & Chaudhri, N. Effects of dopamine receptor antagonism and amphetamine sensitization on sign- and goal-tracking after extended training. *BioRxiv* 1–12 (2019) doi:https://doi.org/10.1101/848150.
164. Cook, C. D. & Beardsley, P. M. The modulatory actions of dopamine D2/3 agonists and antagonists on the locomotor-activating effects of morphine and caffeine in mice. *Pharmacol. Biochem. Behav.* **75**, 363–371 (2003).
165. Day, J. J., Roitman, M. F., Wightman, R. M. & Carelli, R. M. Associative learning mediates dynamic shifts in dopamine signaling in the nucleus accumbens. *Nat. Neurosci.*

10, 1020–1028 (2007).

166. Gibson, G. D. *et al.* Distinct accumbens shell output pathways promote versus prevent relapse to alcohol seeking. *Neuron* **2**, 1–9 (2018).
167. Bossert, J. M. *et al.* Role of projections from ventral subiculum to nucleus accumbens shell in context-induced reinstatement of heroin seeking in rats. *Psychopharmacology (Berl).* **233**, 1991–2004 (2016).
168. Fuchs, R. A., Evans, K. A., Parker, M. C. & See, R. E. Differential involvement of the core and shell subregions of the nucleus accumbens in conditioned cue-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl).* **176**, 459–465 (2004).
169. Xia, Y. *et al.* Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. *J. Neurosci.* **31**, 7811–7816 (2011).
170. Hu, X. T. & White, F. J. Dopamine enhances glutamate-induced excitation of rat striatal neurons by cooperative activation of D1 and D2 class receptors. *Neurosci. Lett.* **224**, 61–65 (1997).
171. Hopf, F. W., Cascini, M. G., Gordon, A. S., Diamond, I. & Bonci, A. Cooperative activation of dopamine D1 and D2 receptors increases spike firing of nucleus accumbens neurons via G-protein betagamma subunits. *J. Neurosci.* **23**, 5079–5087 (2003).
172. Gaspar, P., Bloch, B. & Le Moine, C. D1 and D2 receptor gene expression in the rat frontal cortex: cellular localization in different classes of efferent neurons. *Eur. J. Neurosci.* **7**, 1050–1063 (1995).
173. Dumartin, B., Doudnikoff, E., Gonon, F. & Bloch, B. Differences in ultrastructural localization of dopaminergic D1 receptors between dorsal striatum and nucleus accumbens in the rat. *Neurosci. Lett.* **419**, 273–277 (2007).
174. Wang, H. & Pickel, V. M. Dopamine D2 receptors are present in prefrontal cortical afferents and their targets in patches of the rat caudate-putamen nucleus. *J. Comp. Neurol.* **442**, 392–404 (2002).
175. Wang, W. *et al.* Regulation of prefrontal excitatory neurotransmission by dopamine in the nucleus accumbens core. *J. Physiol.* **590**, 3743–3769 (2012).
176. Stuber, G. D., Hnasko, T. S., Britt, J. P., Edwards, R. H. & Bonci, A. Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. *J. Neurosci.* **30**, 8229–8233 (2010).
177. Hnasko, T. S. *et al.* Vesicular glutamate transport promotes dopamine storage and glutamate corelease In vivo. *Neuron* **65**, 643–656 (2010).
178. Yoo, J. H. *et al.* Ventral tegmental area glutamate neurons co-release GABA and

promote positive reinforcement. *Nat. Commun.* **7**, 1–13 (2016).

179. Zell, V. *et al.* VTA Glutamate Neuron Activity Drives Positive Reinforcement Absent Dopamine Co-release. *Neuron* **107**, 1–10 (2020).
180. Weiss, F., Lorang, M. T., Bloom, F. E. & Koob, G. F. Oral alcohol self-administration stimulates dopamine release in the rat nucleus accumbens: Genetic and motivational determinants. *J. Pharmacol. Exp. Ther.* **267**, 250–258 (1993).
181. Self, D. W., Barnhart, W. J., Lehman, D. A. & Nestler, E. J. Opposite modulation of cocaine-seeking behavior by D1- and D2-like dopamine receptor agonists. *Science (80-.).* **271**, 1586–1589 (1996).
182. Kim, C. K., Adhikari, A. & Deisseroth, K. Integration of optogenetics with complementary methodologies in systems neuroscience. *Nat. Rev. Neurosci.* **18**, 222–235 (2017).
183. Floresco, S. B. Dopaminergic regulation of limbic-striatal interplay. *J. Psychiatry Neurosci.* **32**, 400–411 (2007).
184. Bamford, N. S., Wightman, R. M. & Sulzer, D. Dopamine's Effects on Corticostriatal Synapses during Reward-Based Behaviors. *Neuron* **97**, 494–510 (2018).
185. Cornish, J. L. & Kalivas, P. W. Glutamate transmission in the nucleus accumbens mediates relapse in cocaine addiction. *J. Neurosci.* **20**, RC89 (2000).
186. Kalivas, P. W., Peters, J. & Knackstedt, L. Animal models and brain circuits in drug addiction. in *Brain Circuits in Addiction* vol. 6 339–344 (2006).
187. Cornish, J. L., Duffy, P. & Kalivas, P. W. A role for nucleus accumbens glutamate transmission in the relapse to cocaine-seeking behavior. *Neuroscience* **93**, 1359–1367 (1999).
188. Egashira, Y., Mori, Y., Yanagawa, Y. & Takamori, S. Development of lentiviral vectors for efficient glutamatergic-selective gene expression in cultured hippocampal neurons. *Sci. Rep.* **8**, 2–7 (2018).
189. Siegel, P. S. & Stuckey, H. L. The diurnal course of water and food intake in the normal mature rat. *J. Comp. Physiol. Psychol.* **40**, 365–70 (1947).
190. Bolles, R. C. The interaction of hunger and thrst in the rat. *J. Comp. Physiol. Psychol.* **54**, 580–584 (1961).
191. Legault, M., Rompré, P.-P. P. & Wise, R. A. Chemical Stimulation of the Ventral Hippocampus Elevates Nucleus Accumbens Dopamine by Activating Dopaminergic Neurons of the Ventral Tegmental Area. *J. Neurosci.* **20**, 1635–1642 (2000).
192. Valyear, M. D. & Chaudhri, N. Context controls the timing of responses to an alcohol-predictive conditioned stimulus. *Behav. Processes* **173**, 1–4 (2020).

193. Taha, S. A. & Fields, H. L. Encoding of palatability and appetitive behaviors by distinct neuronal populations in the nucleus accumbens. *J. Neurosci.* **25**, 1193–1202 (2005).
194. Krause, M., German, P. W., Taha, S. A. & Fields, H. L. A pause in nucleus accumbens neuron firing is required to initiate and maintain feeding. *J. Neurosci.* **30**, 4746–4756 (2010).
195. Gass, J. T., Sinclair, C. M., Cleva, R. M., Widholm, J. J. & Olive, M. F. Alcohol-seeking behavior is associated with increased glutamate transmission in basolateral amygdala and nucleus accumbens as measured by glutamate-oxidase-coated biosensors. *Addict. Biol.* **16**, 215–228 (2011).
196. Meyer, H. C. & Bucci, D. J. Imbalanced Activity in the Orbitofrontal Cortex and Nucleus Accumbens Impairs Behavioral Inhibition. *Curr. Biol.* **26**, 2834–2839 (2016).
197. Wise, R. A. & Bozarth, M. A. A psychomotor stimulant theory of addiction. *Psychol. Rev.* **94**, 469–492 (1987).
198. Di Chiara, G. & Imperato, A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5274–5278 (1988).
199. Di Chiara, G. Drug addiction as dopamine-dependent associative learning disorder. *Eur. J. Pharmacol.* **375**, 13–30 (1999).
200. Wise, R. A. & Robble, M. A. Dopamine and Addiction. *Annu. Rev. Psychol.* **71**, 79–106 (2020).
201. de Wit, H. & Wise, R. A. Blockade of cocaine reinforcement in rats with the dopamine receptor blocker pimozide, but not with the noradrenergic blockers phentolamine or phenoxybenzamine. *Can. J. Psychol.* **31**, 195–203 (1977).
202. Yokel, R. A. & Wise, R. A. Attenuation of Intravenous Amphetamine Reinforcement by Central Dopamine Blockade in Rats. *Psychopharmacology (Berl).* **48**, 311–318 (1976).
203. Wise, R. A., Yokel, R. A. & de Wit, H. Both Positive Reinforcement and Conditioned Aversion from Amphetamine and from Apomorphine in Rats. *Science (80-.).* **191**, 1273–1275 (1976).
204. Fuchs, R. A., Ramirez, D. R. & Bell, G. H. Nucleus accumbens shell and core involvement in drug context-induced reinstatement of cocaine seeking in rats. *Psychopharmacology (Berl).* **200**, 545–56 (2008).
205. Redish, A. D. *The mind within the brain: how we make decisions and how those decisions go wrong*. (Oxford University Press, 2013).
206. Staiger, P. K. & White, J. M. Cue reactivity in alcohol abusers: stimulus specificity and

extinction of the responses. *Addict. Behav.* **16**, 211–221 (1991).

207. Carter, B. L. & Tiffany, S. T. A meta-analysis of cue-reactivity in addiction research. *Addiction* **94**, (1999).
208. Glautier, S., Ph, D. & Drummond, D. C. Alcohol dependence and cue reactivity. *J Stud Alcohol* **55**, 224–229 (1994).
209. Holland, P. C. Occasion setting in Pavlovian conditioning. in *The Psychology of Learning and Motivation* 69–125 (1992).
210. Trask, S., Thrailkill, E. A. & Bouton, M. E. Occasion setting, inhibition, and the contextual control of extinction in Pavlovian and instrumental (operant) learning. *Behav. Processes* **137**, 64–72 (2016).
211. Bouton, M. E. & Swartzentruber, D. Analysis of the associative and occasion-setting properties of contexts participating in a Pavlovian discrimination. *J. Exp. Psychol. Anim. Behav. Process.* **12**, 333–350 (1986).
212. Ross, R. T. & Holland, P. C. Conditioning of simultaneous and serial feature-positive discriminations. *Anim. Learn. Behav.* **9**, 293–303 (1981).
213. Holland, P. C. Temporal determinants of occasion setting in feature-positive discriminations. *Anim. Learn. Behav.* **14**, 111–120 (1986).
214. Holland, P. C. & Lamarre, J. Transfer of inhibition after serial and simultaneous feature negative discrimination training. *Learn. Motiv.* **15**, 219–243 (1984).
215. Holland, P. C. Transfer of negative occasion setting and conditioned inhibition across conditioned and unconditioned stimuli. *J. Exp. Psychol. Anim. Behav. Process.* **15**, 311–328 (1989).
216. Rescorla, R. A. Pavlovian conditioned inhibition. *Psychol. Bull.* **72**, 77–94 (1969).
217. Holland, P. C. Conditioned stimulus as a determinant of the form of the Pavlovian conditioned response. *J. Exp. Psychol. Anim. Behav. Process.* **3**, 77–104 (1977).
218. Weiss, S. J. Stimulus Compounding in Free-Operant and Classical Conditioning. *Psychol. Bull.* **78**, 189–208 (1972).
219. Leising, K. J. & Bonardi, C. *Occasion Setting*. *Behavioural Processes* vol. 137 (American Psychological Association, 1998).
220. Fraser, K. M. & Holland, P. C. Occasion Setting. *Behav. Neurosci.* **133**, 145–175 (2019).
221. Saunders, B. T., O'Donnell, E. G., Aurbach, E. L. & Robinson, T. E. A cocaine context renews drug seeking preferentially in a subset of individuals. *Neuropsychopharmacology* **39**, 2816–2823 (2014).
222. Swartzentruber, D. Blocking between occasion setters and contextual stimuli. *J. Exp.*

Psychol. Anim. Behav. Process. **17**, 163–173 (1991).

223. Fraser, K. M. & Janak, P. H. Occasion setters attain incentive motivational value: implications for contextual influences on reward-seeking. *Learn. Mem.* **26**, 291–298 (2019).
224. McSweeney, F. K. & Swindell, S. Common processes may contribute to extinction and habituation. *J. Gen. pschology* **129**, 364–400 (2002).
225. Trujillo-Pisanty, I., Conover, K., Solis, P., Palacios, D. & Shizgal, P. *Dopamine neurons do not constitute an obligatory stage in the final common path for the evaluation and pursuit of brain stimulation reward*. *Plos One* vol. 15 (2020).
226. Warsi, M., Sattar, S. P., Bhatia, S. C. & Petty, F. Aripiprazole reduces alcohol use. *Can. J. Psychiatry* **50**, 244 (2005).
227. Janiri, L., Martinotti, G. & Nicola, M. Di. Aripiprazole for relapse prevention and craving in alcohol-dependent subjects: Results from a pilot study. *J. Clin. Psychopharmacol.* **27**, 513–549 (2007).
228. Martinotti, G. *et al.* Aripiprazole for relapse prevention and craving in alcohol use disorder: Current evidence and future perspectives. *Expert Opin. Investig. Drugs* **25**, 719–728 (2016).
229. Anton, R. F. *et al.* A randomized, multicenter, double-blind, placebo-controlled study of the efficacy and safety of aripiprazole for the treatment of alcohol dependence. *J. Clin. Psychopharmacol.* **28**, 5–12 (2008).
230. O'Donnell, P. Dopamine gating of forebrain neural ensembles. *Eur. J. Neurosci.* **17**, 429–435 (2003).
231. Bell, K., Duffy, P. & Kalivas, P. W. Context-specific enhancement of glutamate transmission by cocaine. *Neuropsychopharmacology* **23**, 335–344 (2000).
232. Lindesmith, A. R. *Addiction and opiates*. (Aldine Pub. Co., 1968).